EXPERIMENTAL ADENOVIRUS GENE THERAPY FOR CANCER PATIENTS WITH ADVANCED TUMORS

− Translational medicine from basic research to the patient's bedside −

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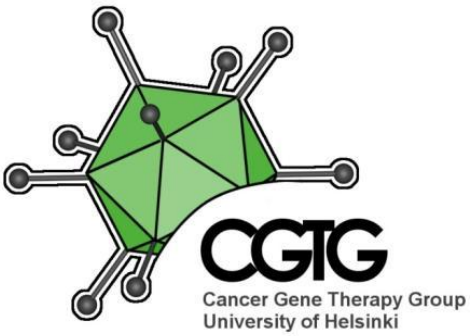

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”Harvoin voi parantaa, usein voi lievittää, aina voi kuunnella.”

- *Vanhaa sanontaa mukaillen*

ABSTRACT

There were 12.7 million new cancer cases in 2008 worldwide and 7.6 million cancer related deaths. Treatment results have improved constantly during the last decades and for many common cancers such as breast cancer and prostate cancer, the 5-year survival rate is higher than 90 %. Unfortunately there are still many cancers, such as pancreatic cancer and lung cancer, for which the 5-year survival is 5 − 10 % or even less. In addition, most advanced cancers lack curative treatments, underlining the need for new effective therapeutics.

Oncolytic adenoviruses provide a new therapeutic approach. Adenoviruses have many features that make them attractive and applicable for cancer treatments. They can be modified to replicate specifically in cancer cells and thereby causing the death of these cells (oncolysis) but sparing healthy cells. Oncolytic adenovirus treatments are also well tolerated and they can be combined with conventional treatments like radiotherapy or chemotherapy to provide better treatment results.

This thesis includes both pre-clinical and clinical sections. In the pre-clinical section we evaluated the combination of replication-deficient adenoviruses and radiotherapy. The purpose was to analyze the mechanism of radiation-mediated upregulation of adenoviral transgene expression. In the clinical section, cancer patients were treated with oncolytic adenoviruses and the safety of the treatments was evaluated as a single or series of treatments. Also, efficacy and immunological responses were evaluated.

Three cancer cell lines were used in the pre-clinical studies: M4A4-LM3 (breast cancer), PC-3MM2 (prostate cancer) and LNM35/eGFP (lung cancer). Cancer cells were exposed to radiation in different temporal and dose schemes and infected 24 h later with various replication-deficient adenoviruses transgenically expressing luciferase or green fluorescent protein reporters. DNA protein kinase inhibitor, heat shock protein 90 inhibitor, and topoisomerase-I inhibitor were used to modify the effect of radiation-induced DNA damage. The transgene expression with or without radiation was evaluated.

Radiation increased adenovirus transgene expression regardless of the transgene, promoter, cancer cell line or radiation dose. We showed that enhancement of transgene expression is mediated through genotoxic stress regulation and repair. Radiation did not increase virus transduction or the availability of viral receptors.

One hundred and fifty seven cancer patients with advanced solid tumors were treated with six different oncolytic adenoviruses according to the Advanced Therapy Access Program (ATAP). The safety of adenovirus treatments was monitored by blood chemistry measurements including clinically relevant laboratory values, cytokine measurements and CTCAE criteria analysis for all

detected adverse events. Biological responses in patients were quantified by measuring neutralizing antibodies and detecting viral genomes in the circulation by quantitative real time PCR (qPCR). Elispot analysis for tumor- and adenovirus-specific T-cells was used to identify the immunological activity caused by the treatment. RECIST analysis and tumor markers were applied to treatment efficacy analysis. We also compared the safety and efficacy of a serial treatment scheme, three rounds of virus within 10 weeks, to a single treatment.

Adenovirus treatments were generally well tolerated and the most commonly detected clinical adverse events were of grade 2 or less: the most common being injection or tumor site pain, other pain, nausea or vomiting, fever and fatigue. Six out of 157 treated patients (3.8 %) experienced a grade 4 adverse event. Serious adverse events were seen in 11 patients (7.0 %), but no treatment related deaths occurred. According to RECIST analysis disease control (= stable disease or better) was seen in 40.0 – 74.0 % of patients and there was stabilization or a decrease in tumor markers for 23.1 – 70.6 % of patients. Serial treatment was as well tolerated as single treatment, but results suggested a better median survival even though statistical significance was not reached.

The synergistic mechanisms of radiotherapy and adenoviruses have been poorly understood and these results provide new molecular information explaining the synergy. Radiation can increase the expression of adenoviral transgenes, which can be used for therapeutic benefit. The analyses of patient treatments show that oncolytic adenovirus therapy is well tolerated and promising evidence of efficacy was seen. Serial therapy seems to be more effective perhaps due to immunological activation and these findings represent a good justification for forthcoming clinical trials.

TIIVISTELMÄ

Maailmanlaajuisesti vuonna 2008 todettiin arviolta 12.7 miljoonaa uutta syöpätapausta sekä 7.6 miljoonaa syöpäkuolemaa. Syövän hoitotulokset ovat kehittyneet tasaisesti viime vuosien aikana ja nykyisin Suomessa 5-vuoden suhteellinen elossaololuku kaikki syöpätyypit huomioituna on yli 60 %. Monen yleisen syövän, kuten rinta- tai eturauhassyövän suhteellinen elossaololuku on jo yli 90 %. On kuitenkin edelleen monia syöpiä, kuten haima- ja keuhkosyöpä, joiden ennusteessa ei ole tapahtunut merkittävää parantumista ja 5-vuoden elossaololuvut ovat jopa alle 5 – 10 %. Samoin levinneisiin syöpiin ei ole edelleenkään tarjolla mitään parantavia hoitomuotoja.

Tarve uusille ja tehokkaille hoitomenetelmille on selvä. Onkolyyttiset adenovirukset ovat eräs lupaava hoitomuoto. Näitä adenoviruksia on muokattu siten, että ne tuhovat spesifisesti syöpäsoluja (onkolyysi) säästäen terveitä soluja. Lisäksi moniin perinteisiin syöpähoitoihin verrattuna adenovirushoidon aiheuttamat haittavaikutukset ovat hyvin siedettyjä ja adenoviruksia on mahdollista myös yhdistää esimerkiksi sädehoidon tai solunsalpaajien kanssa.

Tämä väitöskirjatyö koostuu sekä prekliinisistä että kliinisistä tutkimustuloksista. Prekliinisessä osuudessa tutkittiin soluviljelymalleilla sädehoidon ja adenovirusgeeniterapian yhteisvaikutuksia. Säteilyn tiedetään lisäävän adenovirusten siirtogeenien ilmentymistä ja tarkoitus oli selvittää tähän vaikuttavia tekijöitä. Väitöskirjan kliinisessä osuudessa syöpäpotilaita hoidettiin erilaisilla onkolyyttisillä adenoviruksilla ja ensisijainen tarkoitus oli tutkia hoidon turvallisuutta. Turvallisuutta on vertailu myös sarjahoidon ja kertahoidon välillä. Toissijainen tavoite oli arvioida hoitojen mahdollista tehoa ja hyötyä potilaille sekä tutkia hoidon synnyttämää immunologista vastetta.

Prekliinisissä tutkimuksissa käytettiin kolmea solumallia: M4A4-LM3 (rintasyöpä), PC-3MM2 (eturauhassyöpä) ja LNM35/eGFP (keuhkosyöpä). Syöpäsolut säteilytettiin erilaisissa koeasetelmissa ja infektoitiin useilla erilaisilla lisääntymiskyvyttömillä adenoviruksilla 24 h säteilyn jälkeen. Tutkimuksessa mitattiin säteilyn vaikutusta adenoviruksen siirtogeenien ilmentymiseen ja siirtogeeneinä käytettiin lusiferaasia sekä GFP:tä (green fluorescent protein). Lisäksi säteilyn aiheuttamia DNA vaurioita muokattiin käyttämällä DNA-proteenikinaasiestäjää, topoisomeraasi-I-estäjää sekä lämpösokkiproteiiniestäjää (HSP90). Siirtogeenien ilmentymistä säteilytetyissä soluissa verrattiin käsittelemättömiin soluihin.

Säteily lisäsi adenovirusten siirtogeenien ilmentymistä riippumatta solulinjasta, siirtogeenistä, siirtogeenin promoottorista tai sädeannoksesta. Tulokset osoittavat, että säteilyn aiheuttamien DNA-vaurioiden ja siirtogeenien ilmentymisen välillä on yhteys ja vaurioiden korjausmekanismit liittyvät

ilmiöön. Säteily ei kuitenkaan lisännyt adenovirusten tunkeutumista syöpäsoluihin eikä myöskään vaikuttanut adenovirusreseptorien ilmentymiseen syöpäsoluissa.

Kliinisessä osuudessa 157 syöpäpotilasta hoidettiin kuudella eri onkolyyttisellä adenoviruksella kokeellisen hoito-ohjelman mukaisesti (ATAP = Advanced Therapy Access Program). Potilaiden laboratorioparametrejä sekä sytokiinipitoisuuksia seurattiin hoitojen aikana mahdollisten haittavaikutusten arvioimiseksi ja lisäksi kaikki havaitut haittavaikutukset luokiteltiin CTCAE-kriteeristön mukaan. Hoitojen biologista vastetta mitattiin neutraloivilla vasta-aineilla virusta kohtaan sekä mittaamalla viruksen genomin määrää verenkierrossa hoidon jälkeen qPCR-menetelmällä. Hoitojen aiheuttamaa immunologista T-soluvastetta määritettiin Elispot-menetelmällä. Virushoidon tehoa arvioitiin RECIST-luokituksella sekä mittaamalla vastetta syöpämerkkiaineissa. Sarjahoitoa, joka tarkoittaa kolmea virushoitoa 10 vk kuluessa, verrattiin sekä turvallisuuden että tehon näkökulmasta kertahoitoon.

Yleisesti adenovirushoidot olivat hyvin siedettyjä ja tasoa 1-2 olevia haittoja näkyi kaikilla potilailla. Yleisimmät haitat olivat: kipu kasvaimessa tai pistokohdassa, muu kipu, pahoinvointi tai oksentelu, kuume ja väsymys. Kuudella potilaalla 157:stä (3.8 %) havaittiin tasoa 4 oleva haittavaikutus ja vakavia hoitoon liittyviä haittoja raportoitiin kaikkiaan 11 potilaalla (7.0 %). Hoitoon liittyviä kuolemia ei todettu. RECIST luokituksella mitattuna hoitovaste saavutettiin 40.0 – 74.0 %:lle potilaista ja kasvainmerkkiaineilla mitattuna 23.1 – 70.6 %:lle. Sarjahoito ei lisännyt haittavaikutuksia verrattuna kertahoitoon, mutta sillä oli suotuisa vaikutus potilaiden elossaoloaikaan joskaan ero ei ollut tilastollisesti merkittävä.

Adenovirusten ja sädehoidon yhteisvaikutukset *in vitro* tunnetaan puutteellisesti, mutta tuloksemme antavat tärkeää uutta tietoa asiasta. Tulostemme mukaan säteily lisää siirtogeenien ilmentymistä ja tätä voidaan hoidollisesti hyödyntää. Adenovirusgeeniterapiahoidot ovat turvallisia ja lupaavia hoitovasteita saavutettiin useille potilaille. Lisäksi sarjahoito näyttää olevan tehokkaampi ja parantavan potilaiden ennustetta mahdollisesti tehostuneen immunologisen vasteen vuoksi. Kliinisiä kontrolloituja hoitotutkimuksia kuitenkin tarvitaan tulosten varmistamiseksi.

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Hämeenlinnassa, Tammikuussa 2013

Petri Nokisalmi

CONTENT

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Nokisalmi P**, Rajecki M, Pesonen S, Escutenaire S, Soliymani R, Tenhunen M, Ahtiainen L*, Hemminki A*.
Radiation-Induced Upregulation of Gene Expression from Adenoviral Vectors Mediated by DNA Damage Repair and Regulation.
Int J Radiat Oncol Biol Phys. 2012 May 1;83(1):376-84. Epub 2011 Oct 20

II Pesonen S, **Nokisalmi P**, Escutenaire S, Särkioja M, Raki M, Cerullo V, Kangasniemi L, Laasonen L, Ribacka C, Guse K, Haavisto E, Oksanen M, Rajecki M, Helminen A, Ristimäki A, Karioja-Kallio A, Karli E, Kantola T, Bauerschmitz G, Kanerva A, Joensuu T, Hemminki A.
Prolonged systemic circulation of chimeric oncolytic adenovirus Ad5/3-Cox2L-D24 in patients with metastatic and refractory solid tumors.
Gene Ther. 2010 Jul;17(7):892-904. Epub 2010 Mar 18.

III **Nokisalmi P**, Pesonen S, Escutenaire S, Särkioja M, Raki M, Cerullo V, Laasonen L, Alemany R, Rojas J, Cascallo M, Guse K, Rajecki M, Kangasniemi L, Haavisto E, Karioja-Kallio A, Hannuksela P, Oksanen M, Kanerva A, Joensuu T, Ahtiainen L, Hemminki A.
Oncolytic adenovirus ICOVIR-7 in patients with advanced and refractory solid tumors.
Clin Cancer Res. 2010 Jun 1;16(11):3035-43. Epub 2010 May 25.

IV Kanerva A[#], **Nokisalmi P**[#], Diaconu I, Koski A, Cerullo V, Liikanen I, Tähtinen S, Oksanen M, Heiskanen R, Pesonen S, Joensuu T, Alanko T, Partanen K, Laasonen L, Kairemo K, Pesonen S, Kangasniemi L, Hemminki A.
Anti-viral and anti-tumor T-cell immunity in patients treated with GMCSF coding oncolytic adenovirus.
Manuscript submitted

* Authors Ahtiainen L. and Hemminki A. have equal contribution in publication I

# Authors Nokisalmi P. and Kanerva A. have equal contribution in publication IV

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

Ad	Adenovirus
AD	Alzheimer's disease
ADA	Adenosine deaminase
ADP	Adenoviral death protein
AE	Adverse event
ATAP	Advanced therapy access program
ALA	α-lactalbumin
ALP	Alkaline phosphatase
ALT	Alanine amino transferase
AST	Aspartate amino transferase
ATM	Ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
BCL-2	B-cell lymphoma 2
BP	Base pair
CA	Carbohydrate antigen
CAR	Coxsackie-adenovirus receptor
CD	Cytosine deaminase
CEA	Carcinoembryonic antigen
CMV	Cytomegalovirus
COX	Cyclooxygenase
CT	Computed tomography
CTCAE	Common Terminology Criteria for Adverse Events
CTL	Cytotoxic T-lymphocyte
DM-1	Myotonic dystrophy locus
dsDNA	double stranded DNA
DNA-PKi	DNA (dependent) protein kinase inhibitor
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
GFP	Green fluorescent protein
GM	Growth media
GM-CSF	Granulocyte macrophage colony stimulating factor
HLA	Human leukocyte antigen

HRE	Hypoxia response element
HSP	Heat shock protein
HSP90i	Heat shock protein 90 inhibitor
HSPG	Heparan sulfate proteoglycan
HSV	Herpes simplex virus
i.a.	Intra-arterial
IARC	International Agency for Research on Cancer
IDO	Indolamine-2,3-dioxygenase
IFN	Interferon
IL	Interleukin
i.m.	Intramuscular
i.p.	Intraperitoneal
i.pl.	Intrapleural
irRC	Immune-related response criteria
ITR	Inverted terminal repeat
i.t.	Intratumoral
i.v.	Intravenous
LUC	Luciferase
MAPK	Mitogen activated protein kinase
MDR	Multi-drug resistance
MHC	Major histocompatibility complex
MLP	Major late promoter
MOF	Multi organ failure
MRI	Magnetic resonance imaging
MV	Measles virus
ND10	Nuclear domain 10
NDV	Newcastle disease virus
NGF	Nerve growth factor
NIH	National Institutes of Health
NIS	Sodium/Iodide symporter
NOD-LRR	Nucleotide-binding oligomerization domain/leucine-rich repeat
NSCLC	Non-small-cell lung cancer
OTC	Ornithine transcarbamylase
PAMP	Pathogen-associated molecular pattern

PBMC	Peripheral blood mononuclear cell
PET	Positron emission tomography
PNP	Purine nucleoside phosphorylase
PRR	Pattern-recognition receptors
PSA	Prostate specific antigen
RB	Retinoblastoma
RECIST	Response Evaluation Criteria in Solid Tumors
RGD	Arginine-lysine-aspartic acid
ROS	Reactive oxygen species
SAE	Serious adverse event
SCID	Severe combined immunodeficiency
SFDA	(China's) State Food and Drug Administration
SIRS	Systemic inflammatory response syndrome
TK	Thymidine kinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOPO-i	Topoisomerase inhibitor
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
TSP	Tumor specific promoter
VEGF	Vascular endothelial growth factor
VP	Viral particle
WHO	World Health Organization

REVIEW OF THE LITERATURE

1. Introduction

Worldwide, one in eight deaths is due to cancer; cancer causes more deaths than AIDS, tuberculosis, and malaria combined. When countries are grouped according to national economy parameters, cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries (Table 1) (American Cancer Society, 2011). According to a recent World Health Organization (WHO) estimate, cancer has replaced ischemic heart disease as the overall leading cause of death worldwide in 2010 (World Health Organization, 2007). According to estimates from the International Agency for Research on Cancer (IARC), there were 12.7 million new cancer cases worldwide in 2008 and the corresponding estimate for total cancer deaths was 7.6 million (American Cancer Society, 2011). These numbers are estimated to be 21.4 million and 13.2 million in 2030, respectively (American Cancer Society, 2011). In Finland, almost 30,000 new cases were diagnosed and about 11,500 people died due to cancer in 2010 (www.cancerregistry.fi, 2012). The most common cancers worldwide are prostate cancer and breast cancer, for males and females, respectively.

Table 1. Leading causes of death worldwide in developing and in developed countries in 2004 (number of deaths, thousands).

	Worldwide			Developing			Developed		
	Rank	Deaths	%	Rank	Deaths	%	Rank	Deaths	%
Heart diseases	1	8,923	15.1	1	7,342	14.5	2	1,563	19.3
Malignant neoplasms	2	7,424	12.6	2	5,255	10.4	1	2,154	26.6
Cerebrovascular diseases	3	5,712	9.7	3	4,949	9.8	3	757	9.4
Lower respiratory infections	4	4,177	7.1	4	3,910	7.7	4	305	3.8
Perinatal conditions*	5	3,180	5.4	5	3,141	6.2		35	0.4
Chronic obstructive pulmonary disease	6	3,025	5.1	6	2,737	5.4	5	285	3.5
Diarrhoeal diseases	7	2,163	3.7	7	2,148	4.2		14	0.2
HIV/AIDS	8	2,040	3.5	8	2,018	4.0		20	0.2
Tuberculosis	9	1,464	2.5	9	1,448	2.9		15	0.2
Road traffic accidents	10	1,275	2.2	10	1,158	2.3		114	1.4
Diabetes mellitus	11	1,141	1.9		914	1.8	7	221	2.7
Malaria	12	889	1.5		888	1.8		0	0.0
Suicide	13	844	1.4		707	1.4	9	118	1.5
Cirrohsis of the liver	14	772	1.3		655	1.3	10	116	1.4
Nephritis and nephrosis	15	739	1.3		611	1.2	8	126	1.6
All causes		58,772	100.0		50,582	100.0		8,095	100.0

The number zero in a cell indicates a non-zero estimate of less than 500.

* Includes "causes arising in the perinatal period" as defined in the International Classification of Diseases, principally low birthweight, prematurity, birth asphyxia, and birth trauma, and does not include all causes of deaths occurring in the perinatal period.

Source: World Health Organization, The global burden of disease: 2004 update.

Considering these numbers it is notable that approximately 60-70 % of all cancer deaths occur in low- and middle-income countries even though cancer is more common in developed countries. This is probably mostly explained by the larger population and more limited access to healthcare providers in the low- and middle-income countries, but also economic issues such as the costs and availability of effective cancer treatments play a significant role. Thirty percent of all cancers could be prevented by controlling dietary and behavioral risks like high body mass index, low fruit and vegetable intake, lack of physical activity and the use of alcohol and tobacco (www.who.int/cancer, WHO, April 2012).

Furthermore, of considerable importance is the emotional and physical suffering caused by cancer. All of the above descibed issues raise the most profound question in oncology: “When will there be a cure for cancer?” To answer this question is very challenging because cancer is not just one disease but hundreds or even thousands of different disorders that share the common feature of unregulated cell growth that disturbs the balance of human physiology. This complexity makes the development of treatment options difficult; a treatment that is effective for a one type of cancer might be totally ineffective for another type of cancer.

Despite this complexity, significant improvements have taken place during the last decades in the treatment of many cancer types. For example, the 5-year survival rate of prostate and breast cancer is about 90 % or even higher in Finland (Figure 1). Unfortunately, there are also cancers like pancreatic and lung cancer, the prognosis of which remains as poor as 60 years ago (Figure 1). Another issue is the advanced metastatic solid tumors that mostly lack curative treatments. Taken together, new innovations and treatment options are needed.

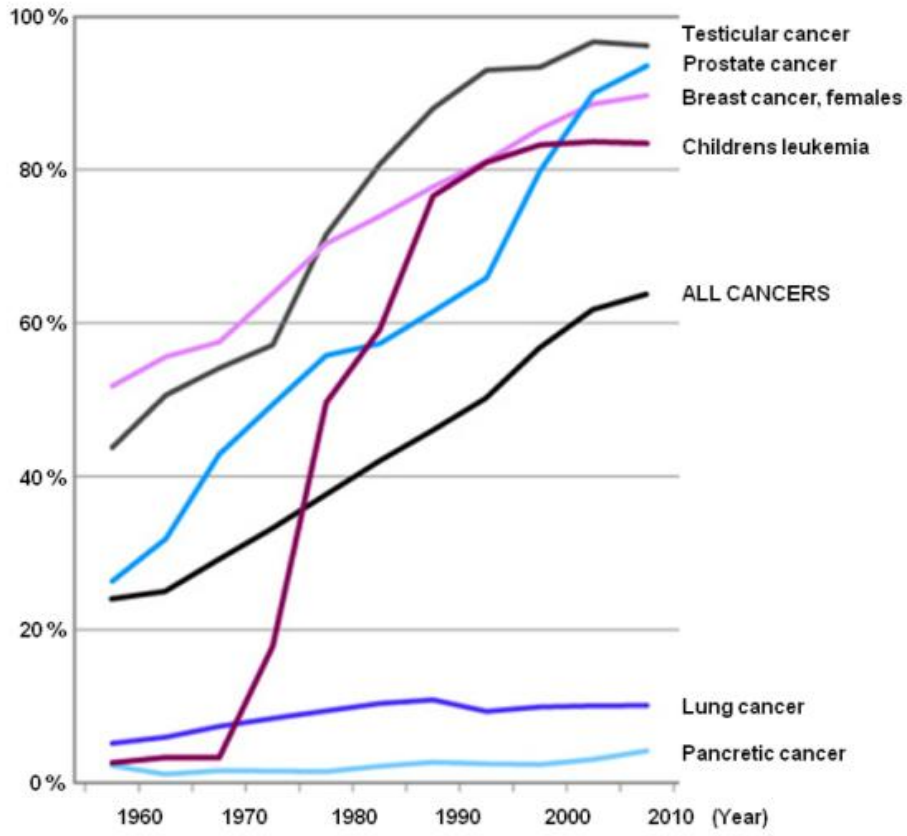

Figure 1. Development of five-year overall survival rate for selected cancers in Finland. Modified from: (Pukkala et al., 2011).

One promising new treatment modality involves the use of oncolytic viruses. Earlier, efforts in adenoviral gene therapy have focused on correction of single gene defects. In the case of cancer gene therapy, this approach is more complicated because cancer cells have various gene defects and distorted cellular functions. Probably, it is because of this diversity why adenoviruses have failed so far in clinical trials to fill the high expectations and only two oncolytic adenovirus products have been approved in China in 2003 and 2005 (Guo and Xin, 2006). Thus, improvement is needed and many challenges in adenoviral cancer gene therapy still exist (Yamamoto and Curiel, 2010): limited tumor transduction reduces the efficacy of treatment, vector spreading within the tumor and human body is limited, and *in vivo* imaging is challenging. Pre-clinical cancer models have also limitations. Firstly, human adenoviruses do not replicate in mouse tissues but they do replicate in transplanted human tumors grown in immunodeficient mice, which distorts pre-clinical results and hampers translational relevance. Secondly, the interactions between adenovirus vectors and human immunology cannot be studied comprehensively in immunosuppressed pre-clinical models. New pre-clinical models such as Syrian hamsters have been introduced to circumvent this limitation, although hamsters are only semi-permissive to adenovirus replication. Thirdly, small experimental

tumors in mice are easier to reach and treat successfully than advanced and disseminated tumors in patients.

Adenoviral cancer gene therapy is still an attractive treatment option for cancer types resistant to conventional treatment. There is the inherent anti-cancer efficacy via oncolysis as such, but in addition oncolytic adenoviruses can be combined with conventional treatments like surgery, chemotherapeutics and radiotherapy to increase the overall efficacy. In addition, adenoviruses can be combined with modern therapies like monoclonal antibodies.

In the preclinical part of this thesis, we show in preclinical models that radiotherapy increases adenoviral transgene expression by modulating genotoxic stress and DNA break repair. This supports the synergy hypothesis of radiotherapy and adenoviruses that might eventually lead to improved treatment efficacy and ultimately better survival of patients.

In the clinical part of this thesis, 157 cancer patients with advanced metastatic tumors were treated according to the Advanced Therapy Access Program (ATAP), with a variety of oncolytic adenoviruses. We show that the treatment is safe and well tolerated. In addition, objective evidence of treatment efficacy was observed. We also present new ideas to enhance adenoviral cancer gene therapy efficacy in patients. These findings create a solid background for forthcoming clinical trials.

2. Gene therapy

Gene therapy can be broadly defined as the use of nucleic acid as a pharmaceutical agent to treat disease. Originally, gene therapy held promise of correcting inherited monogenic diseases by either replacing the defective gene or introducing therapeutic genes to produce therapeutic gene products and proteins in somatic cells (Friedmann and Roblin, 1972). In theory, germ line cells can also be modified by gene therapy, but many ethical and legal aspects limit this approach in human subjects.

In this thesis the focus is on adenoviral cancer gene therapy, but also other human diseases have been suggested and tested as candidates for viral gene therapy approaches. A comprehensive review of this topic is not done, but a few examples are given:

1) Cardiovascular diseases:

Cardiovascular diseases are the leading cause of death worldwide (Table 1). Atherosclerotic diseases are multifactorial disorders characterized by stenosis or total occlusion of arteries causing insufficient perfusion of the target organs. The patients are currently treated with systemic medication, using percutaneous revascularization or bypass surgery. However, many patients cannot be effectively treated by these methods, and vascular gene therapy has been studied as an alternative for these patients. Safety records for cardiovascular gene therapy have been excellent, but low gene transfer efficiency hinders this approach, as reported in several clinical trials (Hedman et al., 2011). Intra-arterial recombinant fibroblast growth factor-2 infusion significantly increased peak walking time at 90 days in patients with moderate-to-severe intermittent claudication (Lederman et al., 2002). Vascular endothelial growth factor (VEGF) gene therapy has been studied for treatment of lower-limb ischemia. Angiography demonstrated that VEGF gene transfer increased vascularity in both adenovirus encoding VEGF and VEGF-Plasmid/Liposome-treated patients (Makinen et al., 2002).

2) Neurological diseases:

Cholinergic neuron loss is a characteristic feature in Alzheimer's disease. Nerve growth factor (NGF) stimulates cholinergic function, improves memory, and prevents cholinergic degeneration. *Ex vivo* NGF gene delivery to patients with mild Alzheimer's disease, by implanting autologous fibroblasts genetically modified to express human NGF into the forebrain suggested improvement in the rate of cognitive decline, and no long-term adverse events occurred (Tuszynski et al., 2005).

Neurotrophic factors can improve the function of degenerating neurons and protect against further neurodegeneration in Parkinson's disease. Intraputaminal injections of adeno-associated virus serotype 2-neurturin (CERE-120) were successfully tested in a phase I trial for patients with Parkinson's disease. The safety profile was extremely good and no clinically significant adverse events were detected during a one year follow-up. Several secondary measures of motor function showed improvement after one year (Marks et al., 2008).

3) Immunology-related genetic diseases:

Adenosine deaminase (ADA) deficiency is a fatal autosomal recessive form of severe combined immunodeficiency (SCID) characterised by impaired immune responses and recurrent infections (Hershfield, 1998). Toxic levels of purine metabolites can cause hepatic, skeletal, neurological and behavioral alterations (Honig et al., 2007; Rogers et al., 2001), as well as sensorineural deafness (Albuquerque and Gaspar, 2004). A hematopoietic stem-cell transplant from a HLA-identical sibling is the treatment of choice, but this is available for only a minority of patients (Buckley et al., 1999; Grunebaum et al., 2006). ADA was the first genetic disorder treated by gene therapy since the beginning of the 1990s when *ADA*-gene-corrected T-cells were used (Bordignon et al., 1995). However, the long-lasting treatment efficacy was limited and all patients required maintenance of polyethylene glycol–modified bovine ADA (the enzyme that corrects metabolic alterations). Furthermore, *ADA*-gene transduced stem cells could not reconstitute the patient's immune system (Blaese et al., 1995; Kohn et al., 1998).

Recently, Aiuti and colleagues treated ten children with SCID caused by ADA deficiency with ADA gene therapy and all patients were alive after a median follow-up of 4.0 years. When they infused autologous CD34+ bone marrow cells transduced with a retroviral vector containing the ADA gene, eight patients did not require enzyme-replacement therapy anymore, their infused bone marrow cells continued to express ADA, and they had no signs of defective detoxification of purine metabolites. Nine patients had immune reconstitution with increased T-cell counts and normalization of T-cell function allowing normal life. Serious adverse events related to ADA gene therapy and autologous bone marrow transplantation included neutropenia, hypertension, central-venous-catheter-related infections, Epstein-Barr virus reactivation and autoimmune hepatitis (Aiuti et al., 2009).

4) Hematological diseases:

Sickle-cell disease is one of the most common hematologic monogenic disorders in the world. Hemoglobin polymerisation, leading to erythrocyte rigidity and vascular occlusion, is essential to the pathophysiology of this disease, although the importance of chronic anemia, haemolysis and vasculopathy has been established (Rees et al., 2010). Lentivirus-mediated gene transfer can correct hematological defects and organ damage in mice with sickle-cell disease (Pawliuk et al., 2001).

5) Psychiatric disorders and addiction:

There is no indication that gene therapy can be used in the treatment of psychiatric patients in the near future. Nevertheless, there are several promising results in experimental neuroscience. Gene therapy approaches have had an effect in animal models of several psychiatric disorders including drug addiction, affective disorders, psychoses and dementia (Thome et al., 2011). Carlezon and colleagues created a herpes simplex virus coding CREB (adenosine 3′,5′-monophosphate response element binding protein) to reduce cocaine addiction in rats. Cocaine regulates the transcription factor CREB in a region that is important for addiction in rats. Overexpression of CREB in this region decreases the rewarding effects of cocaine (Carlezon et al., 1998). Rosenberg and colleagues tested an adeno-associated virus (AAV) gene transfer vector as the delivery mechanism of an anti-cocaine monoclonal antibody (GNC92H2) in mice. Naïve mice exhibited hyperactivity whereas AAV treated mice were completely resistant to the cocaine. This may provide a new therapeutic option to treat cocaine addiction (Rosenberg et al., 2012).

3. Adenovirus

3.1 Adenovirus structure

Adenoviruses are non-enveloped, double-stranded DNA viruses that belong to the family of *Adenoviridae*. Adenoviruses have been characterized extensively since their initial description in the early 1950s (Hilleman and Werner, 1954; Rowe et al., 1953). Based on their ability to agglutinate human erythrocytes, human adenoviruses are divided into seven species (A-G) and all together 53 different serotypes have been identified so far (Smith et al., 2010). In this study we have used adenovirus serotypes 5 and 3 that belong to the species C and B, respectively.

The geometric structure of adenovirus is an icosahedral with twelve protruding fibers from the nucleocapsid corners (nucleocapsid diameter 80-100 nm) (Figure 2). The linear double-stranded DNA inside the capsid is 36,000 base pairs long and the virus is relatively large, having a molecular weight of ~150 MDa. The genome encodes more than 40 different proteins and 13 of these have been shown to be constituents of the virus particle (Lehmberg et al., 1999; Russell, 2009; Stewart et al., 1993). Within the core, the viral genome is condensed in association with proteins V, VII, and X (or μ), and the 5′ termini of the adenovirus DNA is covalently linked to the terminal protein. Surrounding the core is a capsid composed of seven proteins: II, III, IIIa, IV, VI, VIII, and IX (Smith et al., 2010). Main viral capsid proteins are 240 units of hexons and 12 units of pentons.

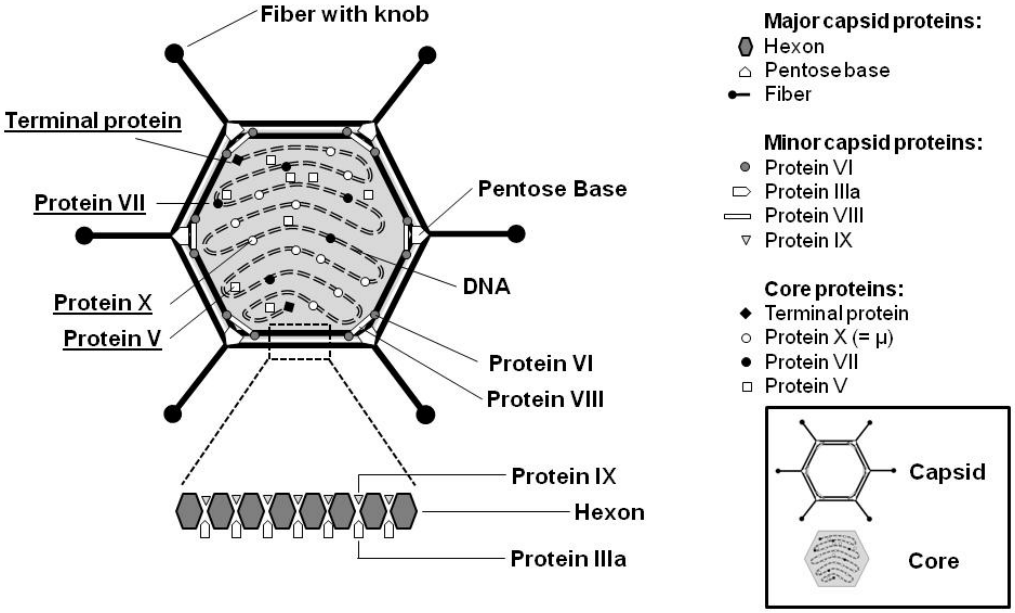

Figure 2. Adenovirus structure. An adenovirus particle is composed of two major structural elements, the outer capsid and the core. Main capsid and core proteins (underlined) are shown. *Modified from:* (Russell, 2000).

Table 2. Adenoviral particle proteins and their main functions. Core proteins underlined.

Protein	Function	References
Protease	Core protein: necessary for production of the infectious virus particle from the procapsid by cleaving the precursors to the structural proteins IIIa, VI, VII, VIII, as well as pTP and precursor of X	(Russell, 2009; Weber, 1976; Webster et al., 1989)
Terminal protein	Core protein: facilitate circularization of the virus genome, needed in viral DNA replication	(Russell, 2009)
Protein II = Hexon	Major capsid protein: most abundant capsid protein (240 hexons in the capsid), there are four kinds of hexons (H1, H2, H3 and H4) based on their different environments	(Russell, 2009)
Protein III = Penton	Major capsid protein: RGD peptide on the penton base interacts with cellular $\alpha_v\beta_3/\alpha_v\beta_5$ integrins, thus facilitating virus internalization and transport into endosomes for further processing	(Russell, 2009)
Protein IIIa	Minor capsid protein: associated with the hexons and pentons to stabilize the virion capsid	(Saban et al., 2006)
Protein IV = Fiber	Major capsid protein: the first virus component to interact with a given tissue and target receptors	(Matthews and Russell, 1995; Russell, 2009)
Protein IVa2	Core protein: binds to DNA and is critical to the packaging process	(Russell, 2009)
Protein V	Core protein: provides a bridge between the core and the capsid	(Russell, 2009)
Protein VI	Minor capsid protein: associated with hexons and pentons to stabilize the virion capsid, rupture the membrane of early endosomes	(Saban et al., 2006; Wiethoff et al., 2005)
Protein VII	Core protein: highly basic and binds tightly to DNA, spreads along the length of the virus DNA	(Russell, 2009)
Protein VIII	Minor capsid protein: associated with hexons and pentons to stabilize the virion capsid, VIII provides a bond between the peripentonal hexons and the rest of the capsid	(Russell, 2009; Saban et al., 2006)
Protein IX	Minor capsid protein: associated with hexons and pentons to stabilize the virion capsid	(Saban et al., 2006)
Protein X (=μ)	Core protein: has properties similar to those found in protamines	(Russell, 2009)

The complete sequence of adenovirus 5 has been published twenty years ago (Chroboczek et al., 1992). The adenoviral genome contains five early transcriptional units (E1A, E1B, E2, E3 and E4), three delayed units or intermediate units (IX, IVa2 and E2 late) and one major late unit that are processed into five late subunits (L1-L5) by alternative splicing (Figure 3). Early genes are transcribed before viral DNA replication and late genes after DNA replication.

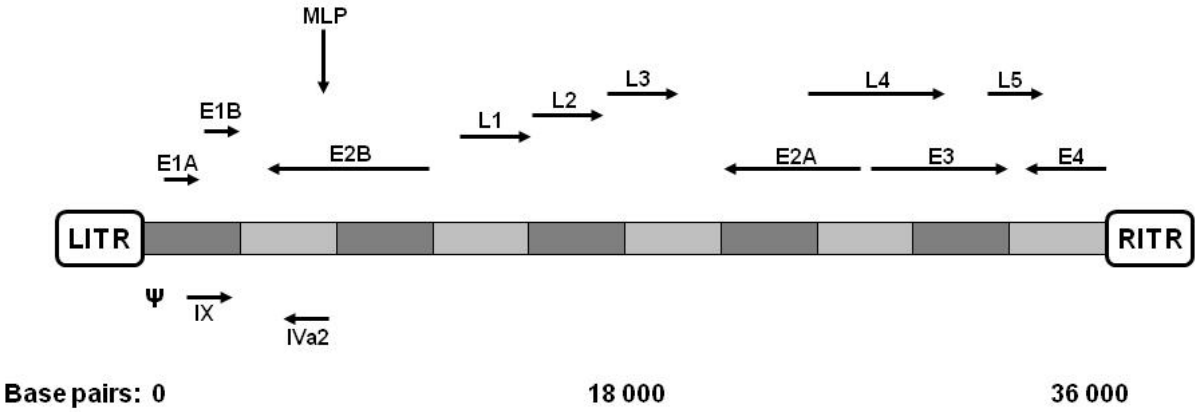

Figure 3. Schematic representation of the adenovirus genome, E = early genes, L = late genes, MLP = major late promoter, LITR = left inverted terminal repeats, RITR = right inverted terminal repeats and Ψ = packaging signal. Packaging signal works as a start point for virus replication whereas inverted terminals repeats are needed for complementary DNA pairing during virus replication. *Modified from:* (Russell, 2000).

The early genes interfere with host cell defense mechanisms and are anti-apoptotic. They also alter cell cycle and modulate cell functions to favor viral replication (e.g. synthesis of DNA-polymerase). Late genes encode structural proteins and inverted terminal repeats are needed in DNA replication where they act as primers. The adenovirus genome also contains viral associated RNAs (VA-RNAs), which are required for efficient translation of viral mRNAs (Thimmappaya et al., 1982).

Table 3. Adenoviral genes and their main functions.

Gene	Function	References
Early genes		
E1A	Primarily functioning in modulating cellular metabolism to make the cell more susceptible to virus replication. Interferes with NF-κB and p53 thereby triggering entry into S phase of the cell cycle. E1A also binds to Retinoblastoma (Rb) family members, releasing E2F transcription factor, thus activating genes required for cell cycling. In addition, E1A proteins inhibit activation of genes induced by IFN and IL-6 and may thus blunt the effect of inflammatory cytokines.	(Anderson and Fennie, 1987; Berk, 2005; Reich et al., 1988; Takeda et al., 1994; Whyte et al., 1988)
E1B	E1B encodes two proteins: E1B-19k and E1B-55k. E1B-19K is homologous in sequence and function to cellular BCL-2. BCL-2 family proteins regulate apoptosis and have both pro-apoptotic and anti-apoptotic proteins. E1B-19K binds to both BAK and BAX (both pro-apoptotic BCL-2 family members), preventing them forming pores in the outer mitochondrial membrane and thus preventing apoptosis. E1B-55K inhibits the transcriptional activation of tumor suppressor protein p53.	(Cuconati and White, 2002; White, 2001; Yew and Berk, 1992)
E2	The E2 gene products are subdivided into E2A and E2B. These provide the machinery for replication of virus DNA and the following transcription of late genes, and this is mediated by interaction with a number of cellular factors. E2A encodes DNA-binding protein, which is required for viral DNA replication and viral DNA is synthesized by the E2B DNA polymerase.	(Hay et al., 1995; Shenk and Flint, 1991; Weitzman and Ornelles, 2005)
E3	E3 gene products provide a compendium of proteins that subverts the host defense mechanisms: (i) adenovirus death protein (ADP) facilitates late cytolysis of the infected cell and thereby releases progeny virus more efficiently, (ii) E3 gp19K is localized in the endoplasmic reticulum membrane and binds the MHC class I heavy chain and prevents transport to the cell surface, where it would be recognized by cytotoxic T-lymphocytes (CTLs). E3 gp19K also inhibits expression of MHC class I and (iii) some E3 proteins inhibit TNF apoptosis.	(Bennett et al., 1999; Russell, 2000; Tollefson et al., 1996)
E4	The gene products derived from the E4 cassette (termed orfs 1 – 6/7) mainly facilitate virus mRNA metabolism (sometimes in association with E1B gene products) and provide functions to promote virus DNA replication and shut-off of host protein synthesis: orf1 facilitates transformation, orf2 has unknown function, orf3 interacts with E1B 55k, orf4 inhibits E1A activation of E2F, orf6 interacts with E1B 55k facilitating RNA metabolism and orf6/7 modulates E2F activity.	(Goodrum and Ornelles, 1999; Halbert et al., 1985; Russell, 2000; Weigel and Dobbelstein, 2000)
Delayed / Intermediate genes		
IX	IX is a transcriptional activator and stimulates MLP.	(Lutz et al., 1997)
IVa2	IVa2 plays a critical role in the transition from early to late phase and controls the activation of MLP.	(Lutz and Kedinger, 1996)
Late genes		
L1-L5	Encodes structural proteins (L2 penton, L3 hexon and L5 fiber) and proteins for encapsulation and maturation of viral particles in the nucleus. L4 100k protein blocks host translation and promotes viral translation.	(Russell, 2000; Weitzman and Ornelles, 2005)

3.2 Adenovirus transduction pathway and life-cycle

The adenovirus infectious cycle can be divided into two phases. The early phase in a permissive cell can take about 6 – 8 h, while the late phase is normally more rapid lasting 4 – 6 h (Russell, 2000). Typically the whole cycle is completed in 24 – 36 h.

The first or early phase:

- Entry of the virus into the host cell
- The passage of the virus genome to the nucleus, followed by the selective transcription and translation of the early genes

The second or late phase:

- Transcription and translation of the late genes
- Production of structural proteins in the nucleus and the maturation of infectious virus.

The fiber in the viral capsid is the first virus component to interact with the target tissue. The infection occurs via an aerosol transmission into the respiratory, gastrointestinal tract, oropharyngeal or conjunctival epithelium (Russell, 2009). Adenoviruses utilize various receptors (Zhang and Bergelson, 2005). The major receptor for most adenoviruses is the CAR receptor (coxsackie adenovirus receptor), which is a member of the immunoglobulin superfamily and is involved *in vivo* in the formation of tight junctions (Bergelson et al., 1997; Coyne and Bergelson, 2005; Coyne and Bergelson, 2006; Philipson and Pettersson, 2004). Adenoviruses from species A, C, E and F interact with CAR receptors while species B and D utilize other receptors e.g. CD46 (Gaggar et al., 2005; Marttila et al., 2005; Segerman et al., 2003; Segerman et al., 2003; Sirena et al., 2004), CD80 or CD86 (Marttila et al., 2005; Short et al., 2004), sialic acid receptors (Arnberg et al., 2002; Segerman et al., 2003), heparin sulphate glycosaminoglycans (Dechecchi et al., 2001) or desmoglein 2 (Wang et al., 2011).

The primary binding of viral fiber to CAR bends the fiber leading to interaction between cellular $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and a RGD peptide on the penton base (Mathias et al., 1998). This facilitates virus internalization via clathrin-coated vesicles into endosomes, where viral capsid is disrupted (Patterson and Russell, 1983). Interaction with integrins induces also a variety of cellular responses, which are important in modifying the cytoskeletal structure in order to facilitate internalization (Li et al., 1998a; Li et al., 1998b). Later, the endosomal membrane is disrupted and the virus attaches to the nuclear pore complex, followed by DNA injection into the nucleus of the host cell (Berk et al., 2007). The mechanism by which the virus genome is imported into the nuclear pore has been shown to involve components of the nuclear pore complex (Greber et al., 1997) and CRM1, a nuclear

factor exported from the nucleus (Strunze et al., 2005). Probably, the hexons are mostly shed at the nuclear pore and the virus core enters the nucleus (Matthews and Russell, 1998). Thereafter, the viral DNA transcription and replication take place in the nucleus and viral proteins are produced in the endoplasmic reticulum (ER) and transported back to nucleus, where viral particles are assembled. The last the step of adenoviral life cycle occurs when new virions are released into extracellular matrix after the cell lysis. The exact mechanism how adenoviruses trigger cell lysis is still poorly understood (Figure 4).

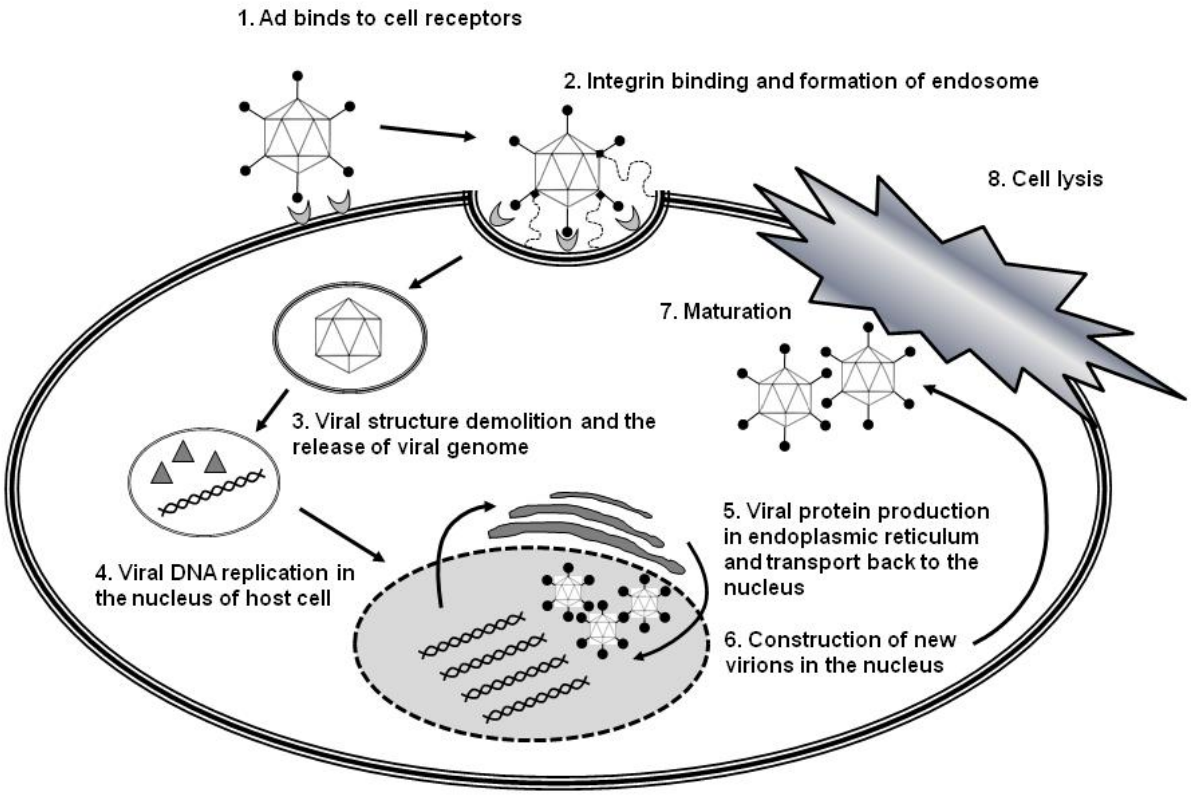

Figure 4. Schematic representation of adenovirus transduction pathway and life-cycle. *Modified from:* (Hakkarainen et al., 2005).

3.3 Clinical manifestations of adenovirus infection

Adenoviruses typically cause mild infections in the upper or lower respiratory tract, gastrointestinal tract, or conjunctiva (Lynch et al., 2011). Different serotypes display different tissue tropism that correlates with clinical manifestations of the infection. Thus, for example species B, C and E mainly cause respiratory disease, whereas species D induces ocular disease. Species F is responsible for gastroenteritis and B2 viruses infect the kidneys and urinary tract (Russell, 2005).

The typical clinical manifestation is tonsillitis with cough, headache and fever. Spontaneous recovery takes place usually within a week (Huovinen et al., 2007). Rare manifestations of adenovirus infections include hemorrhagic cystitis, hepatitis, hemorrhagic colitis, pancreatitis, nephritis, or encephalitis. Adenovirus infections are more common in young children due to immature humoral immunity. Due to childhood exposure, pre-existing antibodies against adenoviruses are very common in adults (e.g. 97 % seroprevalence for group C adenovirus) (Nayak and Herzog, 2010). Epidemics of adenovirus infections may occur in healthy persons in closed or crowded settings (e.g. military recruits) (Lynch et al., 2011). Infection routes include direct contact through the respiratory or gastrointestinal tract, aerosol droplets, oral-fecal route and water (Russell, 2005). The disease is more severe and dissemination is more likely in patients with impaired immunity (e.g. immunosuppressed patients having had an organ transplant). There are no specific drugs against adenovirus infection, but Cidofovir is used for severe adenovirus infections (Lynch et al., 2011). Vaccines have been shown to be very efficacious in reducing the risk of respiratory infection but vaccines have not been available for some time because the only vaccine manufacturer ended production in 1999 (Lynch et al., 2011; Potter et al., 2012). After a 12-year absence, the adenovirus vaccination program for U.S. military recruits was resumed in October 2011 (Potter et al., 2012).

4. Adenoviruses for cancer gene therapy

4.1 A retrospect of oncolytic viruses

The use of viruses in the treatment of cancer resulted from observations that occasionally cancer patients who were affected by an infectious disease went into short periods of clinical remission. These early observations took place in the mid-1800s and in the beginning of 1900s. However, the concept and nature of "virus" was totally unknown in medicine at that time (Kelly and Russell, 2007). Most of the patients whose tumor regression was seen simultaneously with natural virus infection were suffering hematological diseases such as leukemia or lymphoma (Dock, 1904; Pelner et al., 1958). Dock reported in 1896 of a 42-year old female with "myelogenous leukemia" who went into remission after probable influenza infection. It took 37 years to understand that influenza is a viral disease (Kelly and Russell, 2007). In another case report, chicken pox led to regression of lymphatic leukemia in a four year old boy (Bierman et al., 1953). After these early observations, it took about half a century before more rational clinical testing started in the 1950s and 1960s (Table 4). Probably the major cause for this delay was the unclear identity and nature of viruses (Kelly and Russell, 2007).

Because of limited success in the early clinical trials in 1950s and 1960s, many researchers abandoned the concept for years. In the beginning of 1990s new DNA techniques brought viral cancer therapy once again back to light. New DNA techniques made possible to modify viral structure and make them more specific for tumors, reduce undesired adverse events and still maintain their natural potency to kill cancer cells (Kelly and Russell, 2007).

Table 4. Four historically significant clinical trials related to oncolytic viruses. *Modified from:* (Kelly and Russell, 2007)

Year	Virus	Experimental design	Results	Adverse reactions	Reference
1949	Hepatitis B virus	22 patients with Hodgkin's disease. Treated with parenteral injection of unpurified human serum and tissue extracts	14/22 developed hepatitis, 7/22 improved in clinical aspects of disease, 4/22 reduction in tumor size	Fever, malaise, one confirmed death	(Hoster et al., 1949)
1952	Egypt 101 virus (early passage West Nile virus)	34 patients with advanced unresponsive neoplastic disease. Treated i.v. and i.m. injection of bacteriologically sterile mouse brain, chick embryo and human tissue.	27/34 infected, 14/34 oncotropism, 4/34 transient tumor regression	Fever, malaise, mild encephalitis (2 confirmed)	(Southam and Moore, 1952)
1956	Adenovirus (adenoidal-pharyngeal-conjunctival virus = APC)	30 patients with cervical cancer. i.t., i.a. and i.v. injection of tissue culture supernatant.	26/40 inoculations resulted in local necrosis	Vaginal bleeding, infrequent fever (3/30), malaise	(Georgiades et al., 1959)
1974	Mumps virus (wild type)	90 patients with various terminal cancers (gastric, pulmonary, uterine > 50 %). Administered: i.t., i.v., oral, rectal, inhalation of purified human saliva or tissue culture supernatant.	37/90 complete regression or decrease > 50 %, 42/90 < 50 % decrease or growth suppression, 11/90 progressive disease	Reactions for 7/90, bleeding, fever	(Asada, 1974)

4.2 Modified adenoviruses

Adenoviruses can be modified by various ways to make them more suitable for the treatment of human cancer. Possible ways of using adenoviruses in cancer treatment:

1) *Viruses as vectors for functional genes:*
   Adenoviruses transport a functional gene into a target cell to replace the abnormal dysfunctional gene. For example the p53-gene can induce apoptosis of cancer cells (Idema et al., 2007).
2) *Viruses as vectors for therapeutic genes:*
   Adenoviruses transport anti-angiogenic genes which prevents vascular system growth to cancer cells (Zhang et al., 2005). Another option is to use genes that potentiate the effect of other anti-cancer modalities like chemotherapy or radiotherapy.
3) *Viruses as vectors for immunostimulation:*
   Adenoviruses transport GM-CSF (Cerullo et al., 2010) or IL genes (Lee et al., 2006), which activate the human immune system and eventually eradicate the tumor.
4) *Pro-drug therapy and suicide genes:*
   Adenoviruses transport Herpes simplex virus thymidine kinase (HSV-TK) into cancer cells, which converts a harmless pro-drug to an active cytotoxic form in cancer cells, and neighboring cancer cells are destroyed by the so-called bystander effect (Kirn et al., 2002).

5) *Oncolytic adenoviruses:* the adenovirus genome is modified to replicate only in cancer cells and the natural viral replication leads to cancer cell destruction (= oncolysis) (Bischoff et al., 1996).

Approaches 1–4 are based on replication deficient adenoviruses and approach five utilizes replicating adenoviruses. Wild type adenoviruses have many features that make them good tools for cancer gene therapy, but there are also limitations:

Main advantages:

- Adenoviral genome and replication is well characterized
- Both the adenoviral genome and capsid are rather easy to modify
- Adenoviral structure and DNA is stable
- High viral titers (up to 10^{13} VP/ml) can be produced
- Adenoviruses infect both dividing and non-dividing cells
- Adenoviral DNA does not integrate into the host cell genome, resulting in low risk of mutagenesis
- Adenovirus has a high transgene capacity (5.1 – 8.2 kbp)

Main limitations:

- Adenoviral gene expression is transient, which limits clinical use in diseases where long lasting gene expression is crucial
- Natural liver tropism increases liver toxicity and decreases tumor transduction
- Low CAR expression in many tumors reduces tumor transduction
- Human immune system can detect and neutralize adenoviruses rather easily and also pre-existing immunity and antibodies reduces the treatment efficacy
- Adenovirus storage in freezers (lower than -80 °C) and virus treatment preparations might cause practical problems in hospitals because of limited infrastructure and personnel expertise

Due to these limitations, wild-type adenoviruses have to be modified in order to increase tumor transduction and gene expression, decrease liver tropism, and reduce immunogenity. Modifications include both structural changes and viral genome remodeling.

4.2.1 Replication deficient vectors

Replication deficient adenoviruses are used as gene transfer vectors. The capacity to carry foreign DNA is, however, limited as the virus becomes unstable if the genome size exceeds 105 % of the wild-type adenovirus (Bett et al., 1993). This led to development of so called first generation adenoviruses where both E1 and E3 regions are deleted and E1 is often replaced with the desired transgene (Hall et al., 2010). These vectors are replication deficient because limited replication exists despite the absence of E1A (Imperiale et al., 1984). Unfortunately, these vectors induce potent immune responses upon systemic application, limiting their clinical use (Hartman et al., 2008; Muruve, 2004).

Second generation adenoviruses feature deletions in regions E2 or E4 in addition to deleted E1 and E3 regions. These vectors were designed to cause a milder host immune response and thus prolonging transgene expression compared to first generation vectors (Hall et al., 2010). These vectors also feature a higher capacity for transgenes. Adenovirus vectors with deleted E2 or E4 have been constructed (Amalfitano et al., 1998; Dedieu et al., 1997), but it is unclear whether these vectors are any better than first generation vectors since E2 mutation had no effect on the persistence of transgene expression (Engelhardt et al., 1994a; Engelhardt et al., 1994b; O'Neal et al., 1998).

A significant advance in reducing immunogenicity and maximizing transgene capacity was taken when helper-dependent adenovirus vectors were created. These third generation vectors are also known as gutless vectors because all genes except the packaging signal and inverted terminal repeats are removed (see Figure 3) (Fisher et al., 1996).

4.2.2 Replicating vectors

The efficacy of replication deficient adenoviruses in cancer therapy was low and data from clinical trials was not satisfying (Rein et al., 2006). To address this problem, conditionally replicating adenoviruses were created. In this approach, the therapeutic effect is not achieved only by transgene expression but the natural viral replication *per se* causes the lysis of cells. However, these viruses have to be targeted carefully to make sure that cell lysis takes place only in cancer cells (oncolysis) but not in normal healthy cells. The transcriptional- and transductional targeting methods are therefore applied (see section 4.3).

Replication also enables improved transgene expression leading to improved treatment efficacy. In theory, selective replication in cancer cells leads to a situation where virus progeny will spread into new neighboring cancer cells or metastases through the vascular system until all cancer cells are

destroyed. Overall, treatment responses in cancer patients have been quite modest in clinical trials. Thus, it may questionable if viral spreading really happens *in vivo* in physiological conditions.

Probably the most studied replicating oncolytic adenovirus is ONYX-015 (also known as dl1520), which is an E1B deleted adenovirus targeted to replicate selectively in p53-deficient cancer cells (Bischoff et al., 1996). However, later it became clear that ONYX-015 is not specific for p53-null cells (Goodrum and Ornelles, 1998). The H101 virus is very similar to ONYX-015 but in addition to the E1B-55k gene deletion it lacks all E3 proteins. H101 (or Oncorine) is one of the first commercial adenovirus based cancer gene therapy products (see also section 4.3.2 and chapter 8) and it was accepted for head and neck cancer therapy in China 2005 (Garber, 2006; Yu and Fang, 2007).

rAd-p53 (or Gendicine) is a recombinant replication-deficient adenovirus encoding human tumor suppressor protein p53. It was the first approved cancer gene therapy product in China 2003 (Guo and Xin, 2006).

4.3 Targeting of adenoviruses

Adenoviral targeting consists of various techniques that increase the viral selectivity of cancer cells, reduce the tropism to normal tissues, hinder replication in normal tissues, decrease side effects, and ultimately enhance the treatment efficacy and safety. Two approaches are currently used (see Figure 5):

- *Transductional targeting:* the virus structure is modified so that viral entry to cancer cells is more specific.
- *Transcriptional targeting:* the viral genome is modified so that viral replication and gene expression occurs only in cancer cells.

4.3.1 Transductional targeting

As described in section 3.2, the importance of CAR in the transduction of many adenovirus serotypes is well established; however, its role in humans is less clear (Hall et al., 2010). Nevertheless, there is a considerable body of evidence indicating that CAR contributes to the uptake of adenovirus species C (e.g. Ad5) and therefore its expression levels in tumors need to be considered when targeted vectors are designed (Hall et al., 2010). Unfortunately, the expression of CAR is down-regulated in many cancers (Li et al., 1999; Okegawa et al., 2001; Rauen et al., 2002; Wesseling et al., 2001a) and low CAR expression also correlates with poor prognosis and aggressive disease (Matsumoto et al., 2005). There are at least four options to overcome this:

1) Fiber pseudotyping:

In this approach the entire adenovirus knob is replaced with its structural counterpart from another adenovirus serotype that binds a cellular receptor other than CAR. Krasnykh *et al.* created the first adenovirus vector chimera that is based on adenovirus 5 but contains an adenovirus 3 knob (Krasnykh et al., 1996). This Ad5/3 vector transduces cells in a CAR-independent manner and has shown efficacy in preclinical models, as for example, in ovarian cancer (Kanerva et al., 2002a) and in malignant glioma (Zheng et al., 2007).

2) Ligand incorporation:

While many cellular proteins are down-regulated during cancer progression, there is also up-regulation of others, in particular specific cell-surface proteins (Hall et al., 2010). This creates a possibility to exploit the up-regulated proteins by re-targeting adenoviral vectors by adding novel attachment molecules (Nicklin et al., 2005). This can be done by adding a ligand into the fiber knob without replacing the original knob. For example, incorporation of an RGD-motif within the HI loop of the fiber knob redirects viral interactions towards cell surface integrins and has shown encouraging preclinical results in malignant glioma (Tyler et al., 2006) as well as in pancreatic cancer (Bilbao et al., 2002). Another example is to use the polylysine motif (pk7) that binds heparan sulfate proteoglycans (HSPG) to enhance transductional efficacy. In a preclinical malignant glioma model the Ad5-pk7 vector achieved 1000-fold increase in transgene expression compared to adenovirus without pk7 (Zheng et al., 2007). However, this approach needs to be introduced with caution because HSPGs are widely expressed also in normal cells.

3) Ligand incorporation to knobless viruses:

This approach arised from observations that fiber-deleted vectors could be produced (Falgout and Ketner, 1988; Von Seggern et al., 1999). One example is an adenovirus, with a knob replaced by human CD40-ligand, enhancing viral transduction into cancer cells where CD40 is widely expressed (Belousova et al., 2003). *In vivo* systemic administration of this virus resulted in hCD40 expression in the pulmonary vasculature of mice (Izumi et al., 2005).

4) Adapter molecules:

The utilization of adapter molecules in not exactly a “sub-category” for transductional targeting because in this approach, the virus remains intact and adapter function is obtained by molecules that cross-link the adenovirus and a specific cell surface structure. However, it is presented here because the goal to improve transduction is the same as in approaches 1–3. This approach also includes new challenges due to the adapter molecule because its effects in

host organs should be studied first and finally the effects of this two-component combination together. This concept was first published by Douglas *et al.* The folate receptor was targeted by an adenovirus with a folate ligand chemically conjugated to an anti-fiber antibody (Douglas et al., 1996). The folate receptor is over-expressed on the surface of a variety of malignant cells, which also makes this approach appealing.

4.3.2 Transcriptional targeting

To achieve adenoviral replication and gene expression selectivity to cancer cells, transcriptional targeting can be employed. Two approaches are widely used: targeting via genetic deletions or utilization of tumor specific promoters.

1) Targeting via genetic deletions (Type I adenoviruses):

In this approach the tumor targeting is achieved by deleting E1A or E1B regions from the adenovirus. The virus cannot replicate in normal cells in the absence of E1A or E1B, but these deletions are compensated by the carcinogenic mutations in cancer cells leading to cancer cell specific replication.

The prototype for transcriptionally targeted adenovirus is previously mentioned ONYX-015 (see section 4.2.2). ONYX-015 has deletions in the gene coding E1B-55k which inhibits tumor suppressor gene p53 allowing ONYX-015 to replicate selectively in p53-deficient cancer cells (Bischoff et al., 1996; White, 2001; Yew and Berk, 1992). p53 is a tumor suppressor protein that has a central role in cell cycle control and its dysfunction is related to most cancers (Kumar et al., 2008). p53 receives inputs from cell stress and abnormality sensors; if the degree of damage to the genome is excessive, p53 stops cell cycle progression until damages are repaired or it triggers apoptosis if damages are too severe to be repaired (Hanahan and Weinberg, 2011). ONYX-015 showed specific replication in p53 mutated cells (Heise et al., 2000; Heise et al., 1999), but the E1B-55k deletion also partly disrupted the cancer cell killing potential (Dix et al., 2001). Also, some cells with normal p53 were permissive for ONYX-015 replication (Goodrum and Ornelles, 1998).

An alternative strategy to create a replicating oncolytic adenovirus is to delete 24 bps in the constant region 2 of E1A. As described earlier (see section 3.1), products of the E1A gene bind to the cellular retinoblastoma protein (Rb), which leads to displacement of the E2F transcription factor (Whyte et al., 1988). The released E2F further transactivates genes responsible for entry into S phase (Whyte et al., 1988). An adenovirus with the 24 bp deletion

does not inactivate Rb and is therefore able to replicate only in cells with a defective Rb pathway. A defective Rb pathway is common in most cancers (Sherr, 1996).

2) Tumor specific promoters (Type II adenoviruses):

Tumor specific promoters (TSPs) can be used to control viral gene expression and limit the expression in cancer cells (Table 5).

Table 5. Selected tumor specific promoters (TSP) and target cancers.

TSP	Type of cancer	Results	Reference
PSA Prostate specific antigen	Prostate cancer	PSA was inserted to drive E1A expression. CN706 virus destroyed large tumors and abolished PSA production in mice.	(Rodriguez et al., 1997)
CEA Carcinoembryonic antigen	Colorectal cancer with liver metastases	CEA promoter-dependent tk gene expression led to significant tumor size reduction in mice without side effects in liver.	(Brand et al., 1998)
α-fetoprotein	Hepatic cancer	E1B55k-deficient adenovirus carrying E1A and attenuated E1B gene driven by the alpha-fetoprotein promoter (Adv-AFP-E1AdB) reduced mouse liver tumors.	(Ohashi et al., 2001)
Chromogranin A	Neuroendocrine cancer	Chromogranin A promoter controls the E1A gene. Ad[CgA-E1A] virus was able to suppress fast-growing human carcinoid tumors in mice.	(Leja et al., 2007)

There are also ubiquitous promoters that take advantage of regulatory elements common in most tumor cells. Compared to conventional TSPs, ubiquitous promoters are active in various cancer types making them more applicable for viral cancer gene therapy. The cyclo-oxygenase 2 (Cox-2) promoter (Wesseling et al., 2001b), E2F-promoter (Tsukuda et al., 2002) and hypoxia response element (HRE) (Binley et al., 2003) are examples of ubiquitous promoters.

4.4 Blood factors and adenovirus biodistribution

The transductional targeting concept is based on the idea that adenovirus biodistribution is mostly determined by receptor distribution. However, adenovirus serotype 5 biodistribution in animals does not directly correlate with the expression of adenovirus receptors (e.g. CAR). Significant levels of liver transduction occur in mouse models after systemic delivery of the virus (Fechner et al., 1999; Lieber et al., 1997; Shayakhmetov et al., 2004). This can lead to liver toxicity (Young and Mautner, 2001). Intravenous delivery is very common in gene therapy trials, although dissemination of adenoviruses to the bloodstream is very rare in nature (Hall et al., 2010).

It has been demonstrated that liver transduction occurs independently of CAR (Alemany and Curiel, 2001; Martin et al., 2003). Blood coagulation factors like factor IX and complement component C4 binding protein have been proposed to bind to the adenovirus surface and form a bridge from Ad5 to the HSPGs and low-density lipoprotein related-receptor proteins of hepatocytes (Hall et al., 2010). Also additional vitamin K-dependent blood factors (Factor X and VII, protein C) may enhance virus transduction in hepatocytes (Parker et al., 2006). This also gives an opportunity to decrease liver transduction by reducing vitamin K-dependent zymogens with warfarin treatment, leading to lower liver toxicity (Parker et al., 2006). Controversial data exist on the interaction of fiber proteins and coagulation factors: reduced liver transduction was shown with short shaft-containing fibers (Ni et al., 2005; Schoggins et al., 2003; Shayakhmetov et al., 2005), but fiberless virus interacted with Factor X similarly as wild-type virus questioning the involvement of fiber proteins (Waddington et al., 2008). Waddington *et al* also demonstrated that the major binding site for Factor X on the Ad5 capsid is the hexon and that this interaction required calcium (Waddington et al., 2008).

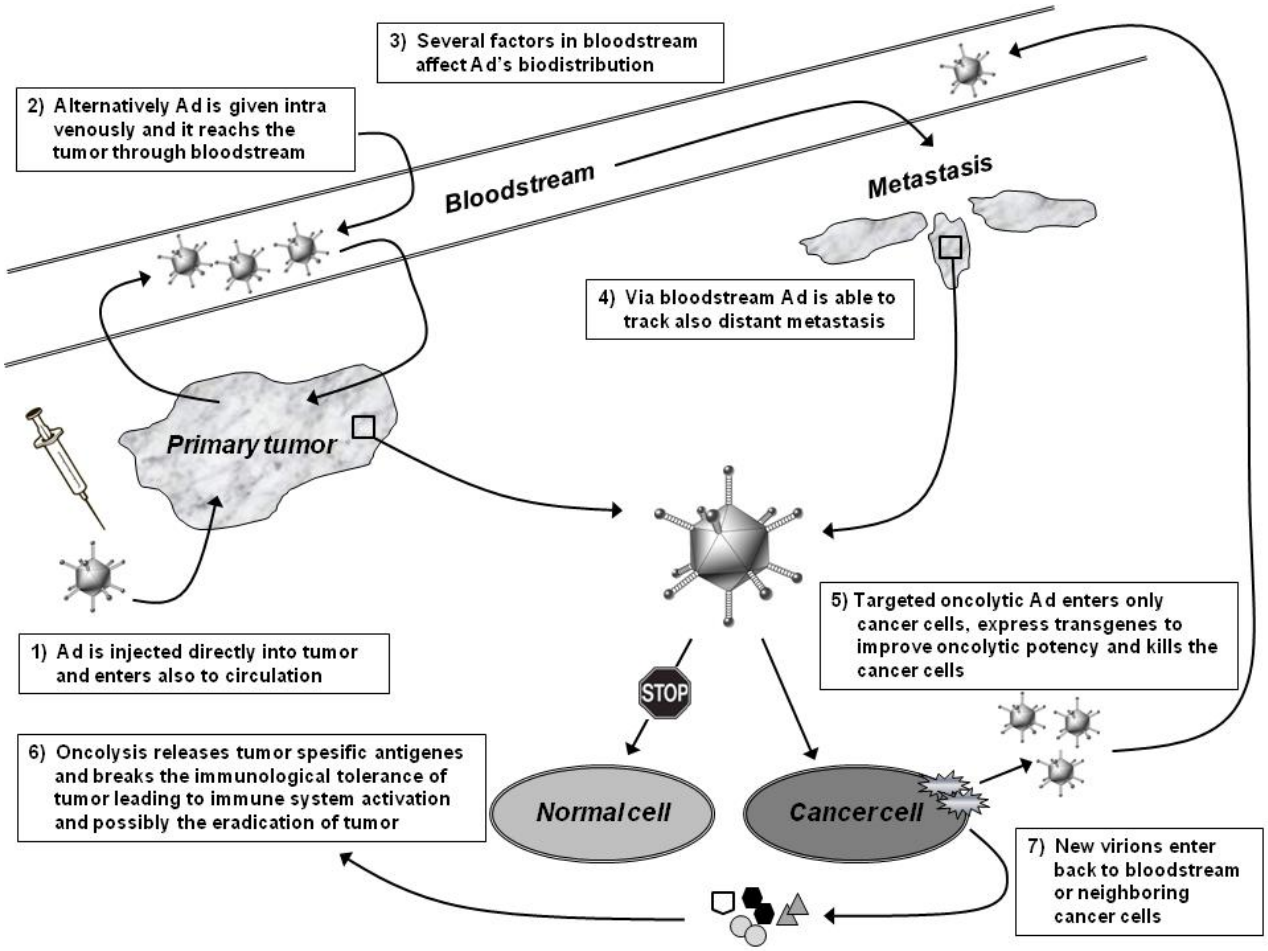

Figure 5. Simplified schematic representation of the main principles of targeted oncolytic adenoviruses in cancer gene therapy.

5. Adenoviruses and radiotherapy

5.1 Introduction to radiotherapy

Many historical landmarks in medicine cannot be dated exactly, but radiotherapy makes an exception. The first treatment with external radiation was given on the 29th of January 1896 in Chicago when a patient with breast cancer was treated (Holsti, 1995). This took place only one year after the discovery of X-rays by Wilhelm Conrad Röntgen. The Nobel Prize in physics in 1901 was awarded to Röntgen "*in recognition of the extraordinary services he has rendered by the discovery of the remarkable rays subsequently named after him"* (www.nobelprize.org., 2012). However, Röntgen himself was not very interested in the biological effects of X-rays.

The first treatment in Finland was given to a sarcoma patient in 1903 in Helsinki (Lahtinen and Holsti, 1997). Since then, remarkable improvements have taken place and modern radiotherapy provides precise techniques to treat successfully various cancer types by using, for example, intensity modulated techniques and arc therapies linked to image-guidance. In addition to more conventional therapies, brachytherapy, boron neutron capture therapy (BNCT) and proton therapy are also used in modern radiation oncology.

5.2 Principles of radiotherapy

The effect of radiotherapy in biology is based on its ability to damage vital cellular components such as DNA or cell membranes. The radiosensitivity varies in different tissues making some tumors more resistant to the effects of radiation, but radiosensitivity can also vary between different cells within the same tumor. The radiosensitivity and -response is the most important factor defining the effect of radiotherapy and it is based on four main principles. *Reoxygenation* due to neo-angiogenesis in tumors repairs hypoxia and oxygen potentiates the radiotherapy response. *Repair* of sub lethal damages in DNA can be repaired more successfully in healthy tissues compared to tumors between fractions. Radiation damage initiates *repopulation* where dead cells are replaced with new ones. Repopulation seems to be more effective in healthy tissues. Cells are also *redistributed* in the cell cycle, rapidly dividing cancer cells are more likely to be in the radiosensitive phase (M-phase or late G_1- and late G_2-phases).

Radiation can damage biological material directly (~30 % of the effect) or indirectly (~70 % of the effect) via radiolysis of water producing reactive oxygen species (ROS). Double strand breaks are lethal to cells but also single strand breaks can be lethal if two such breaks exist simultaneously in close proximity of one another (Lahtinen and Holsti, 1997). ROS oxidise cell membrane phospholipids and fatty acids, which are then hydrolyzed by a number of enzymes (Haimovitz-

Friedman et al., 1994; Hallahan et al., 1994). These enzymes also activate second messengers and further cascades of enzymes within the cytoplasm, typically kinases that can either down- or up-regulate gene expression (Hallahan, 1996). Cell death after radiation therapy occurs by at least three mechanisms: apoptosis, postmitotic cell death and/or necrosis. Apoptosis and postmitotic cell death occurs early, whereas necrosis is a late effect of radiation (Hallahan, 1996). Radiation has a variety of effects on genes that regulate cell proliferation, tissue regeneration, production of cytokines and growth factors, formation of fibrotic tissue, vascular damage response, cell death, radioresistance, cell cycle regulation, signal transduction, and transcription factors (Hallahan, 1996). As radiation damage does not separate normal cells and tumor cells, clinical radiotherapy is a continuous balancing act between tumor control and normal tissue complications. This is a complex endeavor, since the radiotolerance of specific tissues in the body depends on the volume irradiated, total delivered dose, the dose per fraction and the level of acceptable risk (Regine, 2002). Traditionally it has been thought that radiotherapy is a local treatment, so it cannot eradicate and target tumor cells that are outside of the treatment fields or that have metastasized to sites beyond the primary tumor. Nevertheless, there is also evidence indicating that radiotherapy might feature an abscopal effect in which local radiotherapy regress distant metastatic non-radiated tumors via immunological activation (Postow et al., 2012).

Figure 6 summarizes the principle of immediate physical DNA damage due to radiotherapy. The total effect of radiation, however, depends significantly on many other processes as well and overall effects can occur many years later and depend on various complex biochemical and biological mechanisms that are not discussed in detail here.

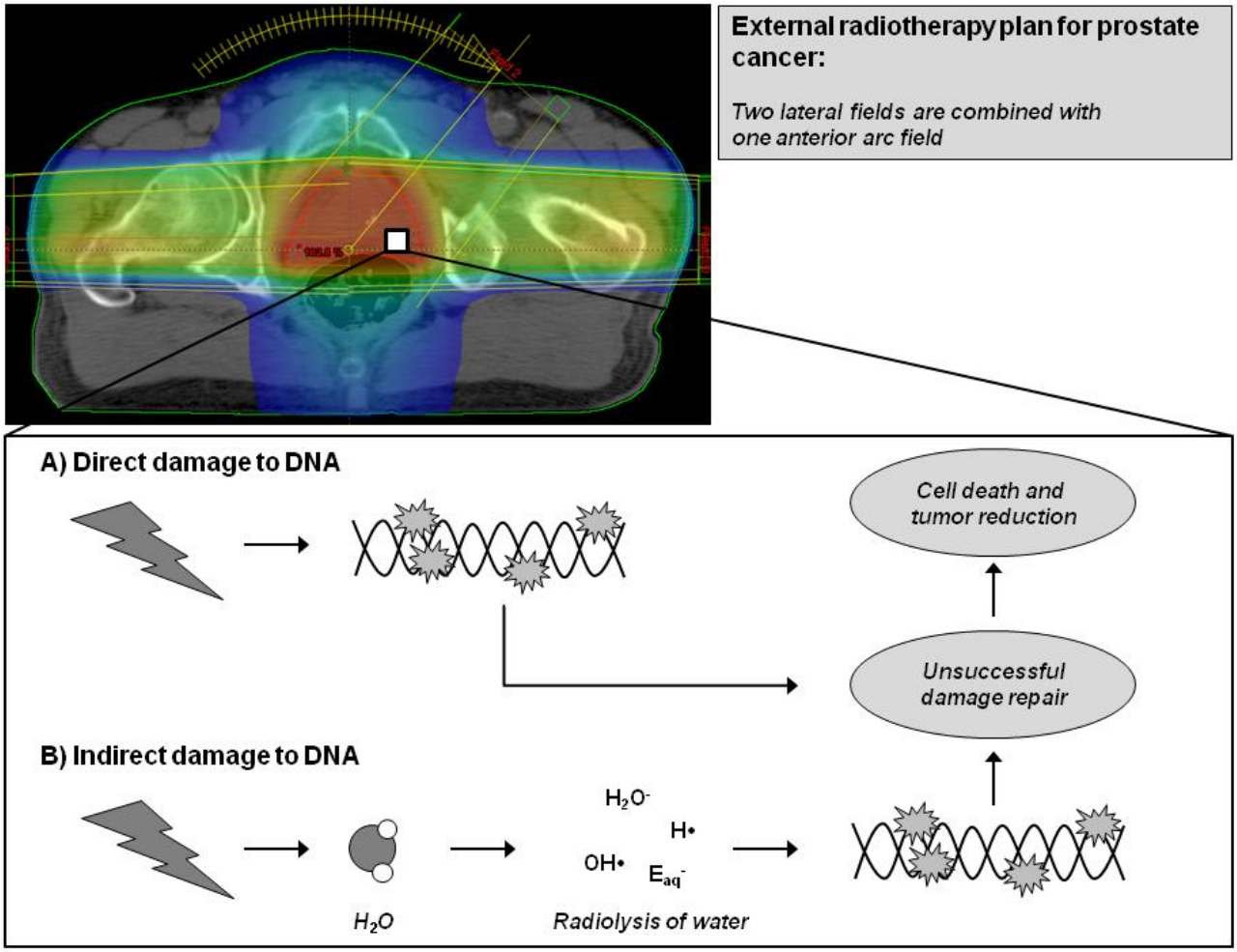

Figure 6. *Upper panel:* An example of an external radiotherapy plan for prostate cancer is shown (transversal CT-image view). In brief, the plan consists of two lateral fields from both sides of the patient and one anterior arc field. In the middle (marked with red outline) is the prostate where the radiation dose is maximal (~ 74 Gy) indicated by the red color. Dose is minimized in surrounding healthy tissues (rectum, bladder and pelvic joints) indicated by green and blue colors.
Lower panel: Schematic representation of the radiation damage to DNA. Radiation damage in tissues is mostly based on the radiolysis of water and the formation of free radicals that interact with DNA. Lethal DNA damage in cancer cells leads to tumor size reduction, whereas in normal cells it may lead to toxicity.

5.3 Combination of adenoviruses and radiotherapy

The combination of radiotherapy and oncolytic adenoviruses is appealing for many reasons. Oncolytic adenoviruses can increase the effect of radiotherapy in a synergistic manner by increasing overall cancer cell killing. Radiotherapy can also increase the expression of therapeutic transgenes from adenoviral vectors and the combination is synergistic in many cases due to the non-overlapping mechanisms of action. Furthermore, the combination does not increase severe adverse events due to the non-overlapping side-effect profile, but the overall number of adverse events might be higher. Finally, both treatments can be applied to various types of cancer.

Several promising trials combining adenovirus-p53 with radiotherapy confirmed the utility of the approach in both head-and-neck cancer and lung cancer (Pan et al., 2009; Swisher et al., 2003). In randomized controlled trials, radiation combined with AdvHSV-tk (adenoviral vector encoding the HSV thymidine kinase gene) increased the survival of patients with glioma or hepatocellular carcinoma (Immonen et al., 2004; Li et al., 2007; Yla-Herttuala, 2008). According to one theory, E1A proteins could sensitize carcinoma cells to radiation (Sanchez-Prieto et al., 1996), but also E1A deleted viruses have shown anti-tumor efficacy with radiation (Lamfers et al., 2002), explained by the fact that not only E1 proteins but also E4 proteins have radiosensitizing features.

The cellular DNA damage repair has been recently linked to these synergy effects. Double strand breaks in DNA can result from exposure to agents such as ionizing radiation and carcinogenic substances, but might be also generated from endogenous sources like meiotic recombination. Mammalian cells have two main pathways for DNA double strand break repair: homologous recombination and non-homologous end-joining (Weitzman et al., 2004). From the DNA damage repair machinery point of view, the double strand DNA breaks and adenoviral double stranded DNA genomes are both perceived similarly.

At late times of infection, amplification of the adenovirus genome presents the cell with as many as 100,000 double stranded DNA ends (Weitzman and Ornelles, 2005). These ends can be sensed as double strand breaks and the host cell responds to this by activating cellular DNA repair machinery (Carson et al., 2003; Stracker et al., 2002). However, it has been shown that only infection with an adenovirus lacking the E4 region induces this cellular DNA damage response forming concatemers of viral DNA, but wild-type virus blocks this signaling through degradation of the Mre11 by the viral E1B55K/E4orf6 proteins (Carson et al., 2003). This suggests that one of the many functions of DNA damage repair machinery is to form an obstacle for wild-type virus infection and especially E4 proteins have a significant role by counteracting this (Weitzman and Ornelles, 2005).

The MRN complex is a key player in the cellular response to DNA damage and recognition of double stranded DNA breaks. The MRN complex contains Mre11, Rad50 and NBS1 proteins

(Stracker et al., 2002). ATM and ATR signal cascades relay the message down-stream of the MRN complex by protein phosphorylation and this signal cascade is also activated during E4 deleted adenovirus infection (Carson et al., 2003). Many cellular DNA repair and checkpoint proteins (e.g. Chk1, Chk2, 53BP1, H2AX, BRCA1, p53 and RPA32) are also substrates for ATM and ATR kinase activity (Kastan and Lim, 2000; Shiloh, 2003).

The E4 region of the virus and especially E4orf3 and E4orf6 are central regulators of the MRN complex. E4orf3 associates with the nuclear matrix and induces the reorganization of nuclear bodies called PML oncogenic domains (PODs) and nuclear domain 10 (ND10) (Doucas et al., 1996). These are large nuclear structures featuring a variety of functions like maintaining genomic stability, repairing DNA damage, controlling transcription and controlling both apoptosis and IFN response (Borden, 2002). E4orf3 rearrange the PODs by an unknown mechanism and this favors viral replication (Maul, 1998). E4orf6 forms a complex with E1B55k protein, and this complex is involved in viral DNA replication, RNA processing and hindering of host protein synthesis (Weitzman and Ornelles, 2005). There is a redundancy between E4orf3 and E4orf6 proteins for their ability to prevent concatemerization and this is achieved by targeting Mre11. E4orf3 mislocalizes Mre11 whereas the E1B55k/E4orf6 complex degrades Mre11 (Stracker et al., 2002). Mre11 degradation by oncolytic adenoviruses is associated also with increased autophagy and the effect was enhanced when viral treatment was combined with radiation (Rajecki et al., 2009). E4orf6 protein may also radiosensitize cells independently of E1B55k and Mre11 (Hart et al., 2005). There is also evidence that both E4orf3 and E4orf6 increase cancer cell killing *in vivo* when transgenically expressed and combined with radiotherapy, making this approach attractive (Liikanen et al., 2010). Both E4orf6 and EIB55k can also independently inhibit the transcriptional activity of p53 and therefore p53 functions in cellular DNA damage are inactivated during viral infection (Cathomen and Weitzman, 2000; Yew and Berk, 1992).

Taken together, the precise reasons for synergy between adenoviruses and radiotherapy are still mostly unknown but the above mentioned aspects are involved. The degradation of the MRN complex by the viral E1B55k/E4orf6 proteins abrogates ATM activation by auto-phosphorylation and subsequent signaling in response to DNA damaging agents like radiation (Weitzman et al., 2004). This contributes to the synergy at least to some extent. Radiation also enhances transgene expression from adenoviral vectors. At a late time point of adenovirus infection, products from the E4 region block the host cell gene expression and in association with E1B55k, E4orf6 directly prevents nuclear export of cellular mRNA (Dobner and Kzhyshkowska, 2001). When promoting late gene expression, the E1B55k/E4orf6 complex and E4orf3 proteins indirectly hinder host cell

gene expression by increasing the expression of 100k protein from L4, which in turn blocks host translation while promoting late viral gene translation (Weitzman and Ornelles, 2005).

In addition to the link between radiation induced DNA damage and adenovirus DNA breaks repair inhibition, there are many other mechanisms that might contribute to the synergy. Radiotherapy has been shown to enhance T-cell trafficking, antigen presentation, and T-cell recognition of tumor cells (Lugade et al., 2005; Reits et al., 2006). This suggests that radiation can increase both the generation of antitumor immune effector cells and their trafficking to the tumor site, which might have a positive impact for immunovirotherapy. However, like many forms of cancer therapies, also radiotherapy can be locally immunosuppressive, killing lymphocytes, and the optimal combination to improve antitumor immune responses will require careful consideration (Prestwich et al., 2008).

6. Immune responses in adenoviral infection and cancer therapy

6.1 Host responses to adenovirus infection

Immunity refers to the protection against infectious agents and the immune system is a very delicate collection of cells and molecules that perform this task. Deficiencies in the immune system can lead to various common diseases such as allergies, bronchial asthma, insulin-dependent diabetes mellitus, inflammatory bowel disease, rheumatoid arthritis and malignancies. Also in cancer, immune system functions are compromised allowing tumor growth and metastasis.

The human immune system consists of innate (or natural) immunity and adaptive (or acquired) immunity. Innate immunity is mostly mediated by cells that are always present in body and are called into action immediately in response to infection, but also immunologically inactive cells such as epithelial cells in the respiratory tract can produce anti-inflammatory agents in infection (e.g. Type I interferons in viral infection). The major components of innate immunity are: epithelial barriers, phagocytic leukocytes (neutrophils and macrophages), natural killer (NK) cells and the complement system. The response of innate immunity is fast but less specific.

Adaptive immunity is more powerful against infectious agents due to its ability to adapt, expand and generate potent, more specific mechanisms to eliminate harmful subjects. The major types and components of adaptive immunity are: humoral immunity created by B-lymphocytes that produce soluble antibody proteins and cell-mediated (or cellular) immunity generated by T-lymphocytes (Kumar et al., 2008).

The understanding of the immune response to adenovirus infection in cancer gene therapy is crucial for three reasons: the rapid clearance of adenoviruses by immunological defense mechanisms decreases the treatment efficacy (Otake et al., 1998; Worgall et al., 1997), the strong immune response to infection might cause severe life-threatening adverse events (Raper et al., 2003), and adenovirus induced immune response can be used for therapeutic benefit in immunovirotherapy. Several aspects of modified adenovirus biology (e.g. genomic deletions and capsid modifications) differ from the biology of wild-type adenovirus infection, therefore the knowledge of wild-type adenovirus biology cannot always be directly applied to adenoviruses used in cancer gene therapy (Muruve, 2004). The roles of both innate immunity and adaptive immunity to adenovirus infection are discussed below.

6.1.1 Innate immunity in adenovirus infection

The first line of defense against adenovirus infection in nature is epithelial cells in respiratory- and gastrointestinal tracts. However, in the context of cancer gene therapy, where adenoviruses are often administered through the intravascular route, the endothelial cells of blood vessels perform this same function, but in nature this situation is rare and less is known how adenoviruses are able to penetrate the endothelial lining. Mechanisms after intratumoral injection are also poorly understood.

Adenoviruses induce innate immunity response shortly after transduction. This innate early response occurs as a result of the interaction of the virus with the cell and does not depend on the transcription of viral genes (Russell, 2009). It is been confirmed that interaction of the viral capsid with the host cell is sufficient to activate inflammatory responses (Muruve, 2004). There is also evidence that adenovirus elimination takes place in two phases at least in the liver, where the early phase is mediated by innate immunity and is the dominant mechanism resulting in a 90 % loss of vector DNA during the first 24 hours after infection (Worgall et al., 1997).

The first task of innate immunity is to recognize the virus, which occurs via the nucleotide-binding oligomerization domain/leucine-rich repeat (NOD-LRR) family of proteins and pathogen-associated molecular patterns (PAMPs) using pattern-recognition receptors (PRRs) (Muruve, 2004). The best-described PRR is the Toll-like receptor (TLR) family. The most important TLRs in adenovirus infection are TLR2 on the cell surface, and TLR9 in endosomes (Appledorn et al., 2008; Cerullo et al., 2007). TLR9 recognizes unmethylated CpG sequences in any DNA including adenoviral DNA, and it may be that the adenovirus genome is sufficiently different from the methylated CpG seen in normal cell DNA to signal that foreign DNA is present (Cerullo et al., 2007). Virus recognition activates adaptor proteins including MyD88, TRIF and TRAF6 (Akira, 2006), leading to signal transduction via mitogen-activated protein kinases (MAPKs) and finally to the activation of NF-κB and the transcription of host cytokine genes (e.g. IL-5, IL-6, IL-8, IL-12, TNF-alpha, RANTES, MIP-2) and type I interferons (Girardin et al., 2002; Inohara and Nunez, 2003). This cytokine response controls the infection locally and recruits granulocytes, NK-cells, macrophages to perform cytolytic function and secrete more cytokines to amplify the immune response (Muruve, 2004). Viral antigen presentation of macrophages via class I MHC molecules to $CD8^+$ T-cells is essential for the development of adaptive immunity (Kumar et al., 2008). The complete clearance of intracellular viruses depends on the destruction of infected cells by the effector cells from innate immunity (NK-cells) and adaptive immunity ($CD8^+$ T-cells), but much of the antiviral potential of these cells also reflects their ability to produce antiviral cytokines such as IFN-gamma and TNF-alpha at the site of the infection (Guidotti and Chisari, 2001). Furthermore, IFN-gamma secretion is

important in the development of helper T-cell type I adaptive immune response (Taniguchi et al., 2003).

Taken together, structural components of the virus, both capsid and DNA, play various roles in the induction of the innate immune response to adenovirus infection, and outcomes can vary depending on the virus species and the nature of the infected cell (Russell, 2009).

6.1.2 Adaptive immunity in adenovirus infection

The expression of proinflammatory mediators upon adenovirus infection occurs before significant viral gene transcription, suggesting that the adenovirus capsid triggers this response (Muruve, 2004). After intravenous administration, adenoviruses produce a biphasic production of cytokines: the first course corresponds to innate immunity during the first 24 h and happens with first-generation, helper-dependent and inactivated adenoviruses, whereas the second course corresponds to adaptive immunity after 4-5 days and it depends on viral gene expression (Liu et al., 2000; Muruve, 2004). Thus, the second peak is not seen with helper-dependent or inactivated adenoviruses. Nevertheless, the adenovirus particle *per se* is sufficient to trigger an adaptive immune response without gene transcription inducing adenovirus specific cytotoxic T-lymphocytes (Kafri et al., 1998; Roth et al., 2002).

All nucleated cells express class I MHC molecules on cell surface and display viral antigens to T-cells, thus all virus-infected cells can be detected and eliminated by cytotoxic T-cells (Kumar et al., 2008). However, a resting T-cell needs co-stimulatory signals for full activation and these signals come from a dendritic cell when a T-cell´s CD28-molecule binds to the B7-molecule on the dendritic cell (Arstila, 2007). Only dendritic cells are able to induce the sleeping T-cell response. Unlike in class I, the tissue distribution of class II MHC expressing cells is quite restricted, they are constitutively expressed mainly on antigen presenting cells (APC) such as macrophages, dendritic cells and B-cells. Class II MHC displays antigens derived from protein synthesis outside the cell and present these antigens to $CD4^+$ helper T-cells activating them (Kumar et al., 2008). Also, B-cells need two signals for activation, the first comes from antigen and the second comes from T-cells that also produce crucial cytokines for B-cells maturation. Finally, the process that was initiated by dendritic cells turns into dialogue between T- and B-cells, which supports both T- and B-cell activation, maturation and function (Arstila, 2007). A complex set of interactions between the innate and the adaptive immune system results in the activation of $CD4^+$ and $CD8^+$ T-cells and B-cells (Seiler et al., 2007). T- and B-cells also create a memory after primary infection, which is long-lasting and consequently, response to re-infection is faster and more specific.

6.2 Cancer immunology

It was thought that the immune system constantly searches and destroys pre-malignant cells in the body in the absence of external therapeutic intervention and thus prevents the formation of malignant clinical tumors. This concept is more than 100 years old and was presented by Nobel Laureate Paul Ehrlich 1909 (Dunn et al., 2004). This theory is partly controversial, because cancer cells originate anyway from the human´s normal cell populations and because of this, the immune response against these cells is more or less inadequate for tumor eradication. However, tumors have structures that could wake up the immune system, such as mutated proteins encoded by oncogenes or malignant transformation associated proteins. There is also clear evidence that the immune response can improve the prognosis of cancer patients. This is supported by the historical evidence of viral infections (see section 4.1), but probably the most profound piece of evidence is the so-called "graft-versus-leukemia" phenomena, where transplanted allogenic stem cells can destroy remaining leukemia cells and prevent the relapse (Kolb, 2008). It is essential to gain an efficient immune response towards tumors and for most cancers this is not currently possible. Monoclonal antibodies provide one approach to overcome this limitation and many immunotherapies have been approved for cancer (Finn, 2008). Table 6 shows some currently available immunotherapy agents that are approved by the Food and Drug Administration (FDA).

The original concept described by Erhlich was difficult to study, but later when the field of immunology developed and experimental testing became possible, the concept acquired a new name "immunosurveillance" (Burnet, 1970). Most recently, it has been proposed that the immune system not only protects the host against tumor development but also, by selecting for tumors of lower immunogenicity, has the capacity to promote tumor growth. These dual effects have refined the term "immunosurveillance" into "immunoediting" (Dunn et al., 2002). There are three cornerstones in the concept that are denoted the 'three Es' of cancer immunoediting (Dunn et al., 2004). In the first *Elimination* phase the immune system tries to eradicate the developing tumor. If this happens successfully it represents the complete immunoediting process without progression to the subsequent phases. In the second *Equilibrium* phase the host immune system and any tumor cell variant that has survived the elimination phase enters into a dynamic equilibrium, where Darwinian selection takes place: the "best" cancer cell variants carry mutations which make them resistant to immune attack. This is probably the longest phase. In the final *Escape* phase tumor cell variants selected in the equilibrium phase now can grow in an immunologically intact environment.

Table 6. Current clinically approved immunotherapy treatments and prophylactic vaccines. *Modified from:* (Finn, 2008; Mellman et al., 2011)

Treatment	Indication	Principle of action or type of therapy
Allogenic bone marrow transplantation		
	leukemias and lymphomas	The graft-versus-leukemia effects involve the direct killing of tumor cells by donor lymphocytes, together with the subsequent induction of broader innate and adaptive reactions
Prophylactic vaccines		
Inactivated HBV antigen (e.g. Engerix-B)	Hepatic cancer	Prevention of cancers of viral origin: prevention of hepatitis B virus and human papilloma virus infection, respectively
HPV serotype proteins (Cervarix, Gardasil)	Cervix cancers	
Cell based therapies		
sipuleucel-T (Provenge)	Advanced prostate cancer	Comprises an incompletely characterized, complex mixture of peripheral blood mononuclear cells supplemented with a cytokine and tumor-derived differentiation antigen
Monoclonal antibodies		
		Generally: antagonizing oncogenic pathways, opsonizing tumor cells, triggering antibody-dependent cellular cytotoxicity or phagocytosis, apoptosis induction
Trastuzumab	Breast cancer, gastric cancer	Her2-receptor
Panitumumab	Colorectal cancer with wild-type KRAS	Her1-receptor
Cetuximab	Colorectal cancer, Head & Neck cancer	epidermal growth factor receptor
Bevacizumab	NSCLC, colorectal and breast cancer	vascular endothelial growth factor receptor
Rituximab, Ibritumomab tiuxetan, Tositumomab)	Non-Hodgkin´s B-cell lymphoma	CD20 B-cell surface antigen
Ofatumumab	Chronic lymphocytic leukemia	CD20 B-cell surface antigen
Brentuximab vedotin	Hodgkin´s lymphoma	CD30 cell surface antigen
Gemtuzumab ozogamicin	Acute myeloid leukemia	CD33 leukemic-cell surface antigen
Alemtuzumab	Chronic lymphocytic leukemia and T-cell lymphoma	CD52 lymphocyte surface antigen
Ibilimumab	Melanoma	CTLA2 antagonist which leads to T-cell activation
Cytokines/Other		
Aldesleukin	Melanoma and renal cancers	interleukin-2 analogue
IFN-α (Intron A, Roferon-A)	Various cancers	Recombinant interferon
TLR7 (Imiquimod)	Basal-cell carcinoma	Toll-like receptor 7 agonist

The immune system can respond to cancer cells in two ways: by reacting against tumor-specific antigens (molecules that are unique to cancer cells) or against tumor-associated antigens (molecules that are expressed differently by cancer cells and normal cells) (Graziano and Finn, 2005). Tumors counteract by suppressing immunity both systematically and in the microenvironment (Figure 7) (Rabinovich et al., 2007). Tumors have various strategies to produce an immunosuppressive environment that favors the growth of tumors. Tumors can produce immunosuppressive molecules such as TGF-β that induces helper and cytotoxic T-cell arrest, inhibits T-cell effector functions (cytotoxicity and IFN-γ production) and recruits/differentiates $CD4^+$ T-cells into T-regs to induce tolerance (Teicher, 2007). They can also produce soluble Fas-ligand that binds to Fas-receptor on effector T-cells to trigger apoptosis (Houston et al., 2003). Tumors secrete immunosuppressive enzyme indolamine-2,3-dioxygenase (IDO) to catabolize tryptophan and cause T-cell anergy through tryptophan depletion (Muller and Prendergast, 2007; Uyttenhove et al., 2003). Tumor cells inhibit MHC-I, and peptide processing and loading on MHC-I to produce suboptimal antigen exposure (Alemany and Cascallo, 2009).

An increase in regulatory T-cells has been observed in the peripheral blood of patients with head and neck cancer, showing that immunosuppression can be also systemic (Chikamatsu et al., 2007). Patients with colorectal cancer and pancreatic cancer have increased numbers of activated granulocytes (Schmielau and Finn, 2001) and myeloid-derived suppressor cells (Nagaraj and Gabrilovich, 2007), both of which suppress tumor-specific T-cells. Overall, the balance between the immunostimulatory and immunosuppressive forces both at the systemic and microenvironment level will determine whether the tumor can grow and progress, or not. Tumor-induced immunosuppression favors malignant tumor formation (Figure 7).

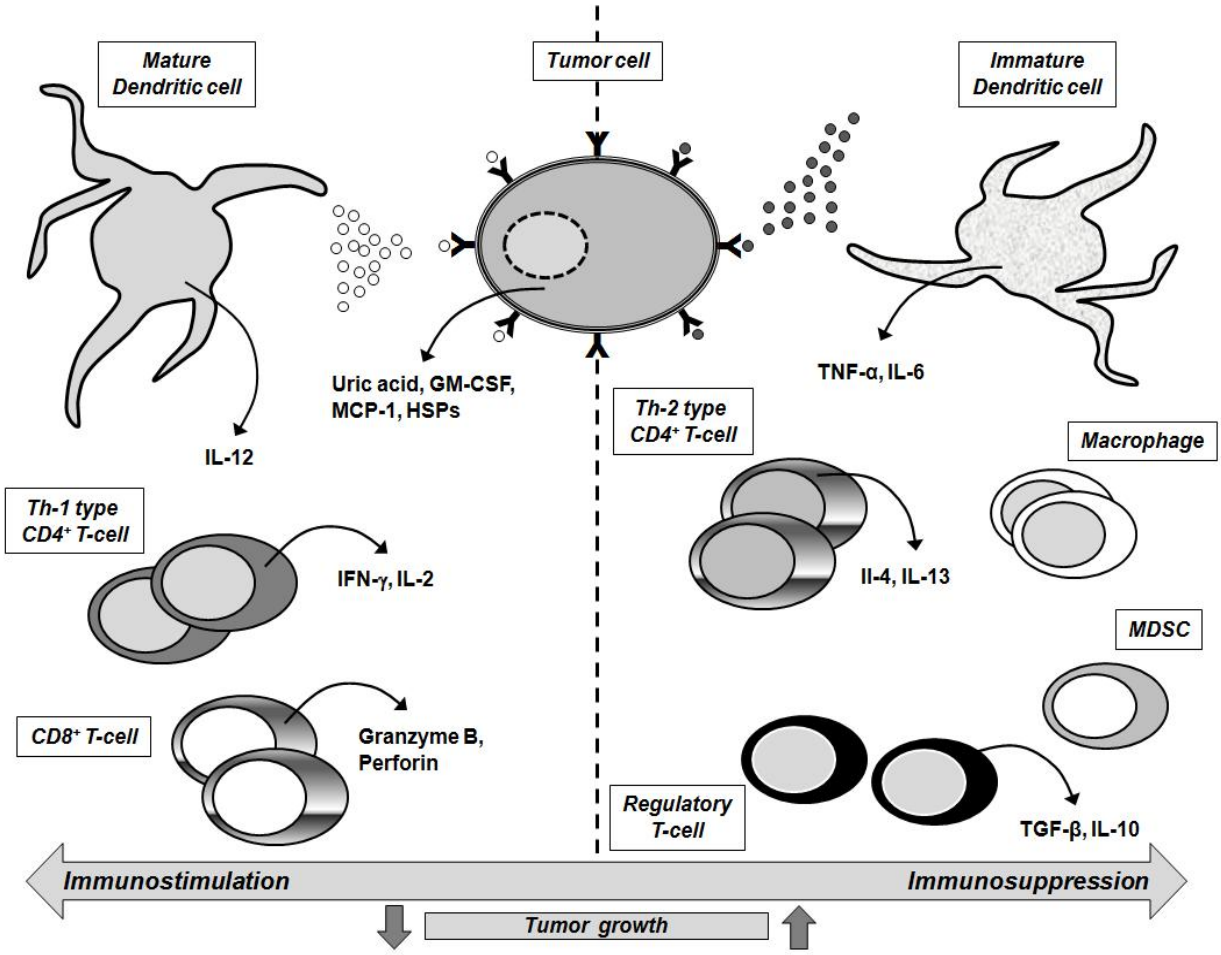

Figure 7. Immunostimulatory and immunosuppressive forces in the tumor microenvironment. A growing tumor secretes both immunostimulatory and immunosuppressive antigens that attract dendritic cells.

Left part (immunostimulation): Dendritic cells take up tumor antigens, mature into IL-12 producing cells, and in the lymph nodes stimulate Th1-type $CD4^+$ T-cells that produce IFN-γ. These cells help expand the population of $CD8^+$ cytotoxic T-lymphocytes that can destroy tumor cells through effector molecules granzyme B and perforin.

Right part (immunosuppression): Another set of tumor antigens promote maturation of a different type of dendritic cell that makes IL-6 and TNF-α and give rise to Th2-type $CD4^+$ T-cells. Those cells make IL-4 and IL-13 and are not effective in tumor rejection. This immunosuppressive environment also promotes generation of regulatory T-cells and macrophages and myeloid-derived suppressor cells (MDSC). At the time the tumor is diagnosed, the balance between the stimulatory and suppressive forces is in favor of tumor-induced suppression. *Modified from:* (Finn, 2008).

6.3 Cancer immunotherapy

Activating the immune system for therapeutic benefit in oncology is known as an *"immunotherapy"*. The cancer immunotherapy history is actually surprisingly long. William Coley, a young surgeon at New York Memorial Hospital, noticed that some patients with sarcoma got better and tumors shrank or even disappeared, when patients got *Streptococcus pyogenes* infection (erysipelas) (Hoption Cann et al., 2003). Coley suspected that somehow the infection was responsible for this miraculous cure and infected his patients with two inactivated bacterial strains *S. pyogenes* and *Serratia marcescens* known as "Coley's toxins" (Coley, 1891; Coley, 1894). His first case was a success, a man with an inoperable sarcoma had complete response and the patient was followed up until his death from a heart attack 26 years later (Nauts et al., 1953).

The passive transfer of anticancer monoclonal antibodies and donor T-cells in the context of allogenic bone marrow transplantation are effective treatments for a variety of hematological and solid malignancies (Dougan and Dranoff, 2009) and these immune treatments have been well-established in oncology for several decades (Mellman et al., 2011). Antitumor immunity suggests at least three sites for therapeutic intervention: promoting the tumor antigen presentation functions of dendritic cells, promoting the production of protective T-cell responses in lymph nodes, and overcoming immunosuppression in the tumor bed (Mellman et al., 2011).

The most important development for cancer immunotherapy recently is ipilimumab, a monoclonal antibody to CTLA4, for late stage metastatic melanoma, either as first line therapy or after relapse (Mellman et al., 2011). In brief, CTLA4 is a negative regulator of T-cells and it binds to members of the B7 family of accessory molecules expressed by dendritic cells and other antigen-presenting cells, thus inhibiting T-cell activation (see also section 6.1.2) (Chambers et al., 2001). Ipilimumab blocks CTLA4 ligation to B7 recruiting T-cells and also blocks regulatory T-cells (Wing et al., 2008).

A phase II trial with ipilimumab showed twofold survival benefit at 12 – 15 months, which was durable after 2.5 years and included a complete response in some patients (Hodi et al., 2010). The frequency of grade 3 or 4 immune-related adverse events was 10 – 15 % and 14 deaths related to the study drugs occurred (2.1 %) (Hodi et al., 2010). In a second randomized trial involving 502 patients with previously untreated metastatic melanoma, the addition of ipilimumab to standard dacarbazine therapy was shown to improve overall survival compared with dacarbazine alone (11.2 months versus 9.1 months) (Robert et al., 2011). Grade 3 or 4 adverse events occurred in 56.3 % of patients (27.5 % with dacarbazine alone), but no treatment related deaths occurred (Robert et al.,

2011). The data above indicates that long-lasting and durable cancer immunotherapy is possible, but the treatment also involves high risks that have to be carefully considered.

6.4 Cancer immunovirotherapy

Oncolytic viruses including adenoviruses may break the immunotolerance of tumors and outbalance tumor immunosuppression mechanisms leading to a significant anti-tumor effect (Alemany and Cascallo, 2009). This concept is called *"immunovirotherapy"*. The concept relies on oncolysis releasing tumor-specific antigens that are taken up by infiltrating antigen-presenting cells for cross-presentation to cytotoxic T-cells for priming of (tumor) antigen-specific immune responses (Diaz et al., 2007; Toda et al., 1999; Todo et al., 1999). The oncolytic process resulting from viral replication is probably not effective enough for tumor eradication, but the triggering of the immune response against the tumor could be long-lasting and more powerful, thereby overriding the natural limitations of viruses. Therefore, oncolytic viruses show the ability to serve as both cytotoxic and immunotherapeutic agents.

One approach in immunovirotherapy is to arm oncolytic viruses with such immunostimulatory molecules that might trigger the desired anti-tumor immune response. Various candidates exist, for example, tumor necrosis factor related apoptosis inducing ligand (TRAIL) (Bernt et al., 2005), IL-12 (Lee et al., 2006), B7-1 (Lee et al., 2006), IL-4 (Post et al., 2007) or GM-CSF (Cerullo et al., 2010). GM-CSF is among the most potent inducers of immunity (Dranoff, 2002) and it activates NK-cells and antigen presenting cells (Andrews et al., 2005; Degli-Esposti and Smyth, 2005). Promising preclinical and clinical results provided data supporting the concept and patients treated with Ad5-d24-GMCSF displayed both anti-tumor and anti-adenovirus $CD8^+$ T-cells immune response without any severe adverse events (Cerullo et al., 2010).

Besides the rapid clearance of adenoviruses as a result of innate immunity, adaptive immunity also creates long-lasting antibodies and memory against adenoviruses. This immunological obstacle could limit the efficacy of adenoviral cancer gene therapy and it originates from the natural frequency of adenovirus infections. Most adults retain Ad-specific cellular memory after childhood exposure and more than 97 % of humans have pre-existing antibodies against group C adenoviruses, but seroprevalence varies for other serotypes (Nayak and Herzog, 2010). These antibodies do not eliminate the virus, but prevent adenovirus binding to cancer cells and promote the phagocytosis of macrophages (Schagen et al., 2004; Yang et al., 1996). One option to circumvent this is to use serotypes with lower natural prevalence.

The immune system can also be manipulated by adjuvant treatments to favor viral replication. Cyclophosphamide is an alkylating agent that can inhibit innate immunity and down-regulate

regulatory T-cells, enhancing the antitumor effect of virotherapy (Di Paolo et al., 2006; Fulci et al., 2006; Ghiringhelli et al., 2004; Ghiringhelli et al., 2007).

Another example of adjuvant therapy is verapamil. Verapamil is a calcium channel blocker commonly used to treat cardiovascular diseases due to its effect of decreasing blood pressure. Verapamil's mechanism of action is understood because of the observation that several viruses induce cell death and progeny release by disrupting intracellular calcium homeostasis (Carrasco, 1995; Ruiz et al., 2000). Gros *et al* showed in pre-clinical models that verapamil enhanced the release of adenovirus from a variety of cell types, resulting in an improved cell-to-cell spread and cytotoxicity and furthermore, the combination an oncolytic adenovirus (ICOVIR-5) with verapamil improved the antitumor activity of ICOVIR-5 in mice (Gros et al., 2010).

7. Overview to other selected oncolytic viruses and their clinical use

Other viruses used in cancer gene therapy trials are the vaccinia virus, herpes simplex virus, reovirus, Newcastle disease virus, and measles virus. More detailed description of the mumps virus is also given because of interesting clinical results, even though it is not currently widely used.

7.1 DNA-viruses

7.1.1 Vaccinia virus

The Vaccinia virus belongs to the *Poxviridae* family and is highly immunogenic, as proven by the production of small pox vaccines and eradication of small pox (Huovinen et al., 2007). The Vaccinia virus features many properties that make it attractive as a cancer therapy. The Vaccinia virus has a high efficiency of infection, its 200 kbp genome allows the insertion of a large amount of recombinant DNA without loss of infectivity, immunostimulatory properties can be used against cancer and the production of conditionally replicative viruses is possible (Eager and Nemunaitis, 2011). Wild-type vaccinia virus does not selectively infect cancer cells, but tumor tropism can be obtained by viral *TK-gene* deletion. The Vaccinia virus replication is possible in cancer cells without the *TK-gene* because cancer cells possess relatively high concentrations of intracellular nucleotide pools (Eager and Nemunaitis, 2011). The *TK-gene* can also be replaced with GM-CSF to improve the anti-tumoral immune response (Mastrangelo et al., 1999).

TG4010 is a targeted immunotherapy agent based on a modified vaccinia virus Ankara coding for MUC1 tumor-associated antigen and IL-2. IL-2 is able to reverse the suppression of the T-cell response caused by the cancer-associated mucin MUC1 (Agrawal et al., 1998). In a phase IIB controlled randomized trial, TG4010 was studied in advanced non-small-cell lung cancer combined with cisplatin and gemcitabine (Quoix et al., 2011). When the combination treatment (74 patients) group was compared to the chemotherapeutics only group (74 patients), six month progression free survivals increased from 35.1 % to 43.2 %. The median overall survival was 10.7 months for the combination group and 10.3 months for the chemotherapy only group (p=0.594).

In another phase II trial TG4010 was tested in the context of clear-cell renal carcinoma combined with IFN-2 and IL-2 cytokines (Oudard et al., 2011). No objective clinical responses occurred, but the median overall survival was 19.3 months (95% CI 11.1–30.2) for all patients (tot. 37 patients) and 22.4 months (95% CI 12.7–32.1) for combination therapy recipients.

TG4010 has also been used for prostate cancer, where it decelerated the PSA doubling time (Dreicer et al., 2009). In a phase II study 13 of 40 patients had more than two fold improvement in

PSA doubling time and 10 patients had their PSA stabilized for over eight months. Overall, the most frequent TG4010-related adverse events have been minor-to-moderate injection site reactions, fatigue and flu-like symptoms in all studies.

7.1.2 Herpes simplex virus

Herpes simplex virus (HSV) is a double-stranded DNA virus that belongs to the *Herpesvirus* family. According to current understanding eight different herpes virus species exist in humans (Huovinen et al., 2007). There are two types of HSV, type I and type II, with rather similar biology and replication. Clinical manifestation of HSV infection includes gingivostomatitis, vulvovaginitis, other genital infections, neonatal infections, conjunctivitis, and encephalitis (Huovinen et al., 2007). HSV type I is used in viral cancer therapy (Varghese and Rabkin, 2002).

Deletions in the viral genome also make HSV specific for cancer cell replication. One modification involved inactivation of viral gene *ICP6*, which encodes the large subunit of ribonucleotide reductase, an enzyme required for viral DNA replication (Goldstein and Weller, 1988). The second gene modification approach consists of deleting another viral gene, the *γ-34.5* gene, which functions as the virulence factor during HSV infection (Chou et al., 1990). A third approach includes *TK-gene* deletion (Martuza et al., 1991). HSV-1 can be used also in suicide gene therapy, initially focused on using the *HSV-TK* gene to convert ganciclovir into toxic metabolites (Springer and Niculescu-Duvaz, 2000) and arm the virus with immunostimulatory molecules such as IL-2, IL-12 or soluble B7-1 (Carew et al., 2001; Toda et al., 1998; Toda et al., 1999). HSV-1 is attractive for cancer therapy, because of the following characteristics (Varghese and Rabkin, 2002):

- it infects many cell types and it is cytolytic by nature
- well-characterized large genome (152 kbp) contains many nonessential genes that can be replaced (up to 30 kbp) with multiple therapeutic transgenes
- a number of nonessential genes are associated with neurovirulence
- antiherpetic drugs are available
- the virus remains as an episome within the infected cell, even during latency, excluding the possibility of insertional mutagenesis

The first replication competent oncolytic herpes virus was described in 1991 for the treatment of malignant gliomas and featured a *TK* mutation (Martuza et al., 1991). Since then, several oncolytic HSV-1 vectors have been studied in clinical trials, for example G207, 1716, NV1020, and OncoVEX$^{GM\text{-}CSF}$. G207 includes *γ-34.5* gene deletion and lacZ insertion thereby disabling the *UL39* gene, and it has been tested for recurrent glioma (Markert et al., 2000). The 1716, deficient in

ICP34.5 protein due to *RL1* gene deletion, has been studied for recurrent glioma and melanoma (MacKie et al., 2001; Rampling et al., 2000). The virulence of NV1020 is substantially weakened by deletions of internal repeat sequences and insertion of an exogenous gene. The NV1020 has been studied for colorectal cancer (Geevarghese et al., 2010; Kemeny et al., 2006) and its safety has been good without any severe adverse events in phase I trials and also some promising therapeutic effects were observed.

OncoVEX$^{GM\text{-}CSF}$ (BioVax, Worcester, MA) is a modified replication-competent HSV-1 with several novel genetic enhancements (e.g. human GM-CSF, intact *TK*) that make it a potent oncolytic and immunogenic vector (Eager and Nemunaitis, 2011). In a phase I study for various solid cancers, the most commonly detected side effects were local inflammation, erythema and fever (Hu et al., 2006). From an efficacy point of view, three out of 26 patients had stable disease, six patients had tumors flattened (injected and/or uninjected lesions) and four patients showed inflammation of uninjected as well as the injected tumor.

Another phase II study of OncoVEX$^{GM\text{-}CSF}$ in metastatic melanoma demonstrated a 26 % objective response by RECIST including uninjected regional and distant metastatic sites (Senzer et al., 2009). Eight complete responses were seen out of 50 treated patients and overall survival was 58 % at 1 year and 52 % at 2 year. Treatment related adverse effects (85 % of patients, all grade ≤ 2) were limited primarily to transient flu-like symptoms (fever 52 %, chills 48 %, nausea 30 %, fatigue 32 %, vomiting 20 %, and headache 20 %).

7.2 RNA-viruses

7.2.1 Reovirus

Reovirus is highly prevalent in the human population, but not associated with any known human disease and is thus considered to be harmless (Russell, 2002). Activated signaling pathways downstream of KRAS or EGFR suppress the activity of double stranded RNA activated protein kinase (PKR), which normally inactivates reovirus replication (Coffey et al., 1998; Strong et al., 1998). Therefore, reovirus is tested in KRAS mutant tumors.

Systemic administration of reovirus has been tested in humans in two phase I monotherapy trials (Gollamudi et al., 2010; Vidal et al., 2008). Commonly observed mild toxicities included fever, fatigue, and headache.

In further clinical trials reovirus has been combined with chemotherapies like gemcitabine (Lolkema et al., 2011). In this study, dose limiting toxicity was detected in three out 16 patients consisting of two asymptomatic grade 3 liver enzyme elevation and one asymptomatic grade 3 troponin I elevation. In another phase I trial, Reolysin (wild-type oncolytic reovirus made by

Oncolytics Biotech) was combined with docetaxel (Comins et al., 2010). Dose-limiting toxicity of grade 4 neutropenia was seen in one patient, but the maximum tolerated dose was not reached. Originally 25 patients were enrolled, but eventually 16 were suitable for response evaluation and among those patients, one complete response and three partial responses were detected (Comins et al., 2010).

7.2.2 Newcastle disease virus

Newcastle disease was the name given to a severe avian disease that occurred in England (Alexander, 1988). This disease has plagued the poultry industry since it was first recognized in 1926 (Lam et al., 2011). Newcastle disease virus (NDV) causes a deadly infection in over 250 species of birds, both domestic and wild, resulting in substantial losses to the poultry industry worldwide (Lam et al., 2011). NDV belongs to the family *Paramyxoviridae* and genus *Avulavirus* (Mayo, 2002).

NDV is interesting from a cancer therapy point of view due to its properties, including being able to replicate itself more rapidly in human tumor cells than in normal cells and cause of oncolytic effects (Lam et al., 2011). NDV can replicate up to 10,000 times better in human transformed cells than in most normal human cells (Pecora et al., 2002; Schirrmacher et al., 1999) and the majority of tumor cell lines can be infected with NDV (Fiola et al., 2006). It has been suggested that NDV's tumor cell activity is based on cancer-specific defects in the interferon pathway, but formal proof is lacking for this hypothesis (Phuangsab et al., 2001). According to Lam *et al* there are many advantages of using NDV in cancer treatment (Lam et al., 2011):

- The virus is not pathogenic to humans
- Ability to bind to the tumor cell surface via its hemagglutinin-neuraminidase glycoprotein
- Replication in tumor cells leads to an enhanced expression of viral antigen on tumor cell surfaces
- Ability of virus to induce synthesis of various cytokines (IFN and TNF), as well as stimulate production of heat shock proteins, adrenocorticotropic hormone, and tissue inhibitor of metalloproteases
- Ability to stimulate TH-cells, CTLs, NK-cells, and macrophages
- Oncolytic activity and rapid growth in cancer cells

The NDV strains that have been most widely evaluated for the treatment of human neoplasms are the nonlytic strain Ulster and the lytic strains MTH68/H, PV-701 and 73-T. PV-701 have shown

good safety and the most commonly detected side effects have been flu-like symptoms, tumor site specific reactions, infusion reactions, pain, leucopenia, thrombocytopenia and diffuse vascular leakages (Laurie et al., 2006; Lorence et al., 2007; Pecora et al., 2002). The same studies have also shown promising anti-cancer potency and no dose limiting toxicity was seen. Laurie *et al* reported four stable disease responses among 16 treated patients with a response duration of six months (Laurie et al., 2006), Lorence *et al* reported four major and two minor responses among 19 patients and six patients survived for at least two years (Lorence et al., 2007).

7.2.3 Measles virus

Measles virus (MV) belongs to the family *Paramyxoviridae*, genus *Morbillivirus* (Huovinen et al., 2007). Typical clinical manifestation includes high fever, cough, conjunctivitis and exanthema among young children followed by lifelong immunity. In Western countries, natural MV infections are mostly eradicated by vaccinations but in developing countries more than one million children die annually due to Measles infections (Huovinen et al., 2007). The MV vaccine strain was isolated in 1954 from the throat washings of an 11-year-old measles patient named David Edmonston and was attenuated by serial tissue culture passages (Msaouel et al., 2009).

The virus infects efficiently on Epstein-Barr virus-transformed B-cell lines, which might explain a dramatic response in an eight-year-old child whose large, untreated retro-orbital endemic Burkitt's lymphoma regressed completely during concomitant measles virus infection (Bluming and Ziegler, 1971). Nevertheless, wild-type MV does not propagate in most cancer cell lines, but certain members of the Edmonston vaccine lineage are potently and selectively oncolytic (Grote et al., 2001; Peng et al., 2001; Peng et al., 2002). The tumor tropism of attenuated MV-Edmonston results from the mutations in the viral attached haemagglutinin protein that enhance its ability to interact with CD46, a regulator of complement activation that is expressed at high levels on the surface of most human tumors (Erlenhofer et al., 2002).

In a phase I trial with intraperitoneal administration of an oncolytic measles virus (MV-CEA virus, which express CEA to permit real time monitoring) for recurrent ovarian cancer, 14 out of 21 patients showed disease stabilization and five patients had significant decreases in CA-125 levels (Galanis et al., 2010). In this study there was no dose-limiting toxicity and basically all side effects were grade 2 or less. There was only one grade 3 toxicity (arthralgia) observed in one patient after a fourth round of virus administration. The most common side effects included fatigue (~34 %), fever (~38 %), anorexia (~29 %), abdominal pain (~24 %), and nausea (~19 %) (Galanis et al., 2010).

Another MV strain, Edmonston-Zagreb vaccine, was studied in five patients with cutaneous T-cell lymphomas (Heinzerling et al., 2005). In this phase I trial, partial regression was observed in four of the intratumorally treated lesions, while one of the treated lesions demonstrated no response. From an immunological perspective, an increase of the IFN-γ/CD4 and IFN-γ/CD8 mRNA ratios, and a reduced CD4/CD8 ratio were observed (Heinzerling et al., 2005). Safety was good also in this study.

A third MV virus used in clinical trials is MV-NIS, which express the NIS gene to concentrate radioisotopes inside cells (Dingli et al., 2003; Dingli et al., 2004). There are at least two open clinical trials ongoing: a trial of intratumoral and resection cavity administration of MV-CEA in patients with recurrent glioblastoma multiforme (NCT00390299) and a second trial of intravenous administration of MV-NIS with or without cyclophosphamide in patients with multiple myeloma (NCT00450814).

7.2.4 Mumps virus

The Mumps virus is a member of the genus *Paramyxovirus* in the family Paramyxoviridae and the typical clinical manifestation of virus infection is parotitis among juveniles (Huovinen et al., 2007). The most promising clinical study performed with Mumps virus was conducted in Japan using a tissue culture adapted strain of mumps virus (Asada, 1974). Ninety patients with various terminal malignancies were treated with minimal toxicity (occasional fever) but the clinical responses were most encouraging. In 37 of 90 patients, the tumor disappeared or decreased to less than half of its original size. Minor regressions were observed in an additional 42 patients (Russell, 2002). For an unknown reason these excellent results did not lead to the further more widespread study of mumps virus as an oncolytic agent (Russell, 2002).

8. Clinical adenoviral gene therapy trials

The first modern oncolytic virus in clinical trial was ONYX-015, E1B deleted oncolytic adenovirus, that was used for recurrent head and neck cancer patients in 1996 with a good safety profile (Ganly et al., 2000). The initial trials tested the efficacy of the ONYX-015 as a single agent and approximately 15-20 % response rate (= stable disease or better) was observed (Ganly et al., 2000; Nemunaitis et al., 2000). No responses were seen for other cancer types such as ovarian, pancreatic and colorectal cancer (Hamid et al., 2003; Hecht et al., 2003; Vasey et al., 2002). Due to the limited activity as a single agent, ONYX-015 has also been studied with chemotherapy and radiotherapy. The response rate was improved from ~20 % to 65 % when ONYX-015 was combined with cisplatin and 5-fluorourasil compared to chemotherapy only (Khuri et al., 2000). The complete response rate in nasopharyngeal cancer patients treated with radiotherapy plus rAd-p53 was 2.73 times higher than the group receiving radiotherapy alone (66.7 % vs. 24.4 %). A six-year follow-up data in the same study showed that combination therapy significantly increased the 5-year locoregional tumor control rate by 25.3 %, but significant results were not observed in 5-year overall survival (Pan et al., 2009). In another phase II trial of head and neck cancer, radiotherapy (70 Gy) plus rAd-p53 improved tumor reduction rate from 62 % to 90 % (Zhang et al., 2003b). Zhang *et al* also reported 2.31 times higher complete response rate two months after treatment with rAd5-p53 combined with radiotherapy (Zhang et al., 2005).

Patients with malignant glioma have been treated successfully with a AdvHSV-tk vector encoding thymidine kinase gene and lacking the E1 and E3 regions. Seventeen patients received AdvHSV-tk gene therapy by local injection into the wound bed after tumor resection, followed by intravenous ganciclovir. The control group of 19 patients received standard care consisting of radical tumor excision followed by radiotherapy. The median survival time increased from 37.7 to 62.4 weeks (Immonen et al., 2004). This vector is also known as Cerepro and is developed by Ark Therapeutics (Kuopio, Finland). However, the Committee for Human Medicinal Products did not approve Cerepro 2008, citing a negative risk-benefit profile due to insufficient efficacy and the risk of hemiparesis and seizures (Sheridan, 2011).

During the last two decades, an increasing number of clinical trials have been conducted with different viruses for various cancers as a single therapy or combined with other regimes. Approximately 140 clinical cancer gene therapy trials with adenoviruses have been conducted since 1996.

In a larger perspective, adenoviral trials have shown very good safety for various diseases and only one treatment related death has been documented (Raper et al., 2003). In that study an 18 year

old male with partial ornithine transcarbamylase (OTC) deficiency died after adenovirus serotype 5 (E1 and E4 deleted containing human OTC cDNA) administration through the hepatic artery (Raper et al., 2003). His clinical course was marked by systemic inflammatory response syndrome (elevated IL-6 and Il-10, but normal TNF-alpha after infusion), biochemically detectable disseminated intravascular coagulation, and multiple organ system failure, leading to death 98 h following gene transfer. This experience pointed out the limitations of animal studies in predicting human responses, substantial subject-to-subject variation, and the need for detailed understanding of the immune response towards adenovirus vectors. Shortly after this event, more concern about gene therapy safety raised the observations of five patients with X-linked SCID who developed T-cell leukemia after retrovirus treatment (Hacein-Bey-Abina et al., 2008). The retrovirus used in this trial contained an enhancer sequence that activated proto-oncogenes leading to T-cell proliferation and leukemia (Hacein-Bey-Abina et al., 2008; Sheridan, 2011). Generally in all reported clinical trials, the most common side effects have been pain at the injection site, fatigue and development of self-limited fever. No treatment related deaths have occurred in cancer trials to our knowledge.

A ten year follow-up data for 146 non-small-cell lung cancer (NSCLC) patients treated with adenovirus gene therapy given as second line treatment in eight different trials during 1995 – 2005 has been also reported (Nemunaitis et al., 2007). A long-term follow-up of reported adverse events showed no grade 3 or grade 4 adverse events. Mean survival was 334 days and the proportion of patients alive at 1 year, 2 years and 5 years were 30 %, 12 %, and 6 %, respectively. When this data was compared to current chemotherapeutic options in the second line for advanced NSCLC, there was no difference in one year survival. One year survivals were 28 – 30 % for pemetrexed, docetaxel and erlotinib (Nemunaitis et al., 2007). Based on this report by Nemunaitis *et al* there are no obvious concerns towards the long-term effect of therapy involving adenoviral vectors in NSCLC patients. Long-term follow-up data for other cancer types has not been reported to our knowledge.

According to the National Institute of Health (NIH) database (April, 2012) there are currently 23 open and active clinical adenoviral cancer gene therapy trials *(www.ClinalTrials.gov)*. Table 7 summarizes the basic characteristics of these trials. Trials with unknown status are excluded.

Table 7. Open adenoviral clinical gene therapy trials for cancer at April, 2012. Data adapted from: www.ClinicalTrials.gov.

Type of cancer	Number of trials	Therapy approach (no.) and design
Bladder cancer	1	Therapeutic transgene: 1 (GM-SCF) Replicative adenovirus: 1
Breast cancer	1	Therapeutic transgene: 1 (GM-CSF) Vaccination/Immunization: 1
Glioblastoma	3	Therapeutic transgene: 1 (TK) Replicative adenovirus: 2
Head and Neck cancer	1	Therapeutic transgene: 1 (Endostatin)
Hepatocellular cancer	1	Vaccination/Immunization: 1
Lung cancer	4	Therapeutic transgene: 4 (p53, CCL21) Infected dendritic cells: 3
Melanoma	6	Therapeutic transgene: 4 (IFN-γ, IFN-α, IL-12, CD40L) Replicative adenovirus: 1 Infected dendritic cells: 1
Mesothelioma	1	Therapeutic transgene: 1 (IFN-α)
Prostate cancer	3	Therapeutic transgene: 3 (PSA, PNP) Vaccination/Immunization: 2
Not specific	2	Therapeutic transgene: 2 (GM-CSF, PNP) Replicative adenovirus: 1

rAd-p53 (or Gendicine) is a replication deficient adenovirus encoding the human tumor suppressor protein p53. China's State Food and Drug Administration (SFDA) approved Gendicine for clinical use in October 2003 and licensed its commercial production in spring of 2004 (Guo and Xin, 2006). SFDA approved Gendicine as a treatment for head and neck cancer based on a small clinical trial, where more patients had complete response with radiotherapy plus Gendicine (64 %) than with radiotherapy alone (19 %) (Guo and Xin, 2006).

Oncolytic virus research took another major step forward in November 2005 when a second cancer gene therapy product Oncorine was approved by SFDA (Garber, 2006). Shanghai Sunway Biotech's genetically modified adenovirus H101 (Oncorine) was approved for head and neck cancer in combination with chemotherapy. This approval was based on the randomized phase III trial findings where cisplatin, 5-fluorourasil (=PF regiment) plus intratumoral injection of H101 or adriamycin, 5-fluorourasil (=AF regiment) plus H101 increased overall response rates compared to chemotherapy alone (Xia et al., 2004). Overall responses were 78.8 % (PF plus H101), 39.6 % (PF alone), 50.0 % (AF plus H101) and 50.0 % (AF alone) (Xia et al., 2004). Survival data was not reported and the approval in Europe and in United States will depend on survival benefit. Survival data was not reported because Chinese regulators at that time based their review on the objective

response rate, not survival. Besides, survival follow-up is difficult because many of Sunway's patients live in isolated rural areas and are never seen by their doctors again. Shanghai Sunway Biotech is also testing H101 in lung cancer and has bought the rights to ONYX-015 (Garber, 2006). Western critics have questioned these results due to lack of available information (Guo and Xin, 2006; Sheridan, 2011).

AIMS OF THE STUDY

The main aims of this thesis are to evaluate in preclinical models the interactions of replication-deficient adenoviruses and radiotherapy (study I), and the safety, efficacy and immunological factors of oncolytic adenovirus treatments in patients with advanced solid cancers (studies II-IV). The specific aims are:

1. To study the combination of replication-deficient adenoviruses and radiotherapy *in vitro* and to analyze the mechanisms of radiation-mediated upregulation of adenoviral transgene expression (I).

2. To study the safety and efficacy of Ad5/3-Cox2L-d24, a triple mutant oncolytic adenovirus with the *COX-2* promoter controlling *E1A* and the serotype 3 knob, in 18 cancer patients (II).

3. To study the safety and efficacy of a novel oncolytic adenovirus ICOVIR-7 in 21 patients with advanced metastatic cancer (III).

4. Our primary goal was to study the safety and anti-tumor effects of three doses of oncolytic adenovirus *versus* single administration in 124 cancer patients. Secondly, we studied T-cells´ responses against tumor and viral epitopes (IV).

MATERIALS AND METHODS

1. Cell lines

Table 8. Human cell lines used in the studies.

Cell line	Description	Source	Original study
LNM35/eGFP	Lung carcinoma cell line, express GFP	T. Takahashi, Honda Research Institute Japan, Saitama, Japan	I
M4A4-LM3	Breast cancer cell line, express GFP	Goodison S., Department of Pathology, University of Florida, FL, USA	I
PC-3MM2	Prostate cancer cell line	Isaiah J. Fidler, M.D. Anderson Cancer Center, Houston, TX, USA	I
293	Embryonic kidney cells transformed with Ad5 E1	American type culture collection, Manassas, VA, USA	I
A549	Human lung adenocarcinoma	American type culture collection, Manassas, VA, USA	II, III, IV

Breast cancer cell line M4A4-LM3 (Goodison et al., 2005) and lung cancer cell line LNM35/eGFP (courtesy of T. Takahashi, Honda Research Institute, Japan) were cultured in RPMI supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Both cell lines express green fluorescent protein (GFP) under the CMV promoter. PC-3MM2 prostate cancer cells are a highly metastatic hormone refractory sub line of PC-3 (courtesy of Isaiah J. Fidler, MD Anderson Cancer Center, Houston, TX). PC-3MM2 cells were cultured in DMEM supplemented with 10 % FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 1 mM sodium pyruvate (Sigma).

2. Viral constructs

2.1 Replication deficient adenoviruses

For large scale production, all replication deficient adenoviruses were propagated in 293 cells. The large scale virus preps were purified by standard cesium chloride gradient centrifugation and VP/ml concentrations were determined by absorbance at 260 nm of purified adenovirus. $TCID_{50}$- assay was used to determine the pfu/ml titer. The presence of correct genes, the absence of wild type contamination and correct viral structure was confirmed by PCR and sequencing. More specific construction conditions are described in original studies referred to in the text with roman numerals (I-IV).

Table 9. Replication deficient adenoviruses used in study I.

Virus	Features	References
Ad5-luc1	Ad5 serotype, deleted E1 region, SV40-luciferase expression cassette in the E1-region under a CMV promoter	(Kanerva et al., 2002a) (Krasnykh et al., 2001)
Ad5/3-luc1	Ad5 serotype, Ad3 knob for enhanced transduction, deleted E1 region, luciferase as a transgene	(Kanerva et al., 2002a), (Krasnykh et al., 1996)
Ad5-luc-RGD	Ad5 serotype, deleted E1 region, RGD-4C motif in the HI-loop to enhance binding to integrins, luciferase as a transgene	(Dmitriev et al., 1998)
Ad5-GL	Ad5 serotype, deleted E1 region, luciferase and GFP as a transgenes	(Wu et al., 2002)
Ad5-pk7-GL	Ad5 serotype, deleted E1 region, seven polylysines at the COOH terminus to enhance binding to HSPG, luciferase and GFP as a transgenes	(Wu et al., 2002)
Ad5-mdr-luc	Ad5 serotype, deleted E1 region, luciferase as a transgene controlled by multi-drug resistance (mdr) promoter	(Bauerschmitz et al., 2008)
Ad5-ala-luc	Ad5 serotype, deleted E1 region, luciferase as a transgene controlled by α-lactalbumin (ala) promoter	(Bauerschmitz et al., 2008)
Ad5-cox2-luc	Ad5 serotype, deleted E1 region, luciferase as a transgene controlled by cyclo-oxygenase (COX) promoter	(Bauerschmitz et al., 2008)
Ad5-vegf-luc	Ad5 serotype, deleted E1 region, luciferase as a transgene controlled by vascular endothelial growth factor (VEGF) promoter	Courtesy of Koichi Takayama

2.2 Oncolytic adenoviruses

All oncolytic adenoviruses used in this study were cloned as described on the previous page except A549 were used for propagation. A more detailed description is found in the original publications.

Table 10. Oncolytic adenoviruses used in studies II, III and IV.

Virus	Features	References
Ad5/3-cox2L-d24	Serotype 5, Ad3 knob, tumor specific cox2L- promoter, 24 bp deletion at E1A region	(Pesonen et al., 2010)
ICOVIR-7	Serotype 5, RGD-motif to enhanced integrin binding, tumor specific E2F-promoter and E2F hairpins for optimal replication, DM insulator for enhanced specificity and Kozak sequence for optimal E1A expression	(Nokisalmi et al., 2010), (Rojas et al., 2009)
CGTG-102 a.k.a Ad5/3-d24-GMCSF	Serotype 5, Ad3 knob, 24 bp deletion at E1A region, GM-CSF transgene in E3 region	(Koski et al., 2010)
Ad5-RGD-d24-GMCSF	Serotype 5, RGD-motif to enhanced integrin binding, 24 bp deletion at E1A region, GM-CSF transgene in E3 region	(Pesonen et al., 2011)
Ad5-d24-GMCSF	Serotype 5, 24 bp deletion at E1A region, GM-CSF transgene in E3 region	(Cerullo et al., 2010)
Ad3-hTERT-E1	Serotype 3, tumor specific hTERT-promoter at E1 region	(Hemminki et al., 2011)

3. *In vitro* methods and protocols (I)

3.1 Cell irradiation

Irradiation was performed with a linear accelerator (Model: Clinac 600C/D, Manufacturer: Varian Medical Systems, Palo Alto, CA, USA) using a 6 MV photon beam (nominal energy) and dose rate 400 MU/min (~ 4 Gy/min). In *in vitro* studies cells were irradiated on tissue culture plates (6- or 24 wells). The plates were arranged on custom made water phantom and irradiated through 1 cm thick plastic phantom bottom and 1 cm thick layer of water in the phantom (gantry angle 180 degrees, field size 40 cm x 40 cm). The source to phantom distance was 100 cm and the absorbed dose was calculated at the depth of cells (2 cm from the bottom of the phantom).

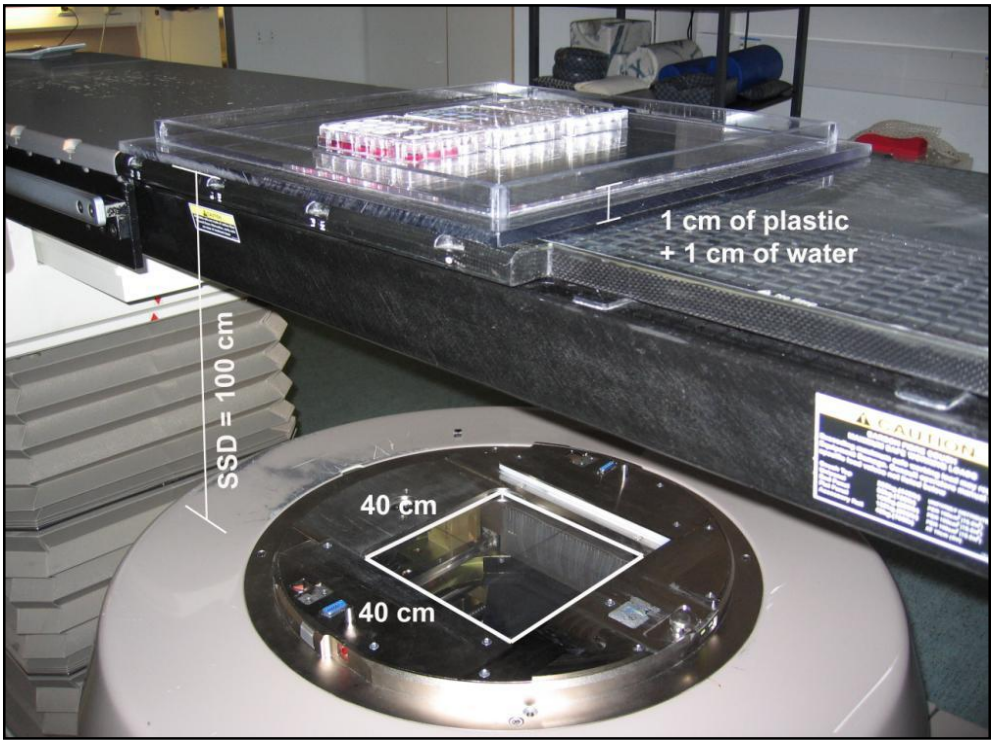

Figure 8. Cancer cell irradiation settings. Tissue culture plates were arranged on plastic water phantom on the treatment table. A maximum treatment field size (40 cm x 40 cm) was used to provide homogenous radiation dose to all tissue culture plates.

3.2 Fluorescence-activated cell sorting analysis

For cell surface receptor analysis, flow cytometric analysis was performed. PC-3MM3 and M4A4-LM3 cells (2 x 10^5) were incubated for 20 min at 4°C with the following primary antibodies at 1:200 dilution: anti-CAR (clone RmcB; Upstate Cell Signaling Solutions/Millipore, Lake Placid,

NY), anti-HSPG (clone F58-10E4; Seikagaku, Falmouth, MA), anti-$\alpha_v\beta_3$ integrin (clone LM609; Chemicon International/Millipore, Temecula, CA), anti-$\alpha_v\beta_5$ integrin (clone P1F6; Chemicon International/Millipore), or anti-CD46 (clone E4.3; BD Biosciences, San Jose, CA). Cells were washed with fluorescence-activated cell-sorting (FACS) buffer (phosphate-buffered saline containing 2 % FBS) and incubated with a 1:200 dilution of phycoerythrin (PE)-labeled secondary antibody (goat anti-mouse polyclonal antibody; BD Biosciences) for 30 min at 4°C. Cells were washed with FACS buffer and analyzed by flow cytometry (FACSCalibur; BD Biosciences) to determine receptor expression levels. Non-stained cells were used as a negative control. The method is adapted from (Hakkarainen et al., 2007).

3.3 Transgene analysis

Transgene activity was determined 24 h after infection (= 48 h after irradiation) if not stated otherwise. For analysis a luciferase assay system (Promega, Madison, WI, TopCount Luminometer, Perkin-Elmer) was used according to the manufacturer's protocol. Flow cytometric analysis was used to determine the proportion of GFP positive cells (gene transfer efficiency) and the intensity of fluorescence in positive cells (level of gene expression).

3.4 Mass spectrometry analysis

HPLC analysis was carried out on an LC Packings system (Amsterdam, Netherlands). This consisted of FAMOS carousel microautosampler connected to an outer pump (Pharmacia LKB, Sweden), Ultimate micro-HPLC pump with UV detector, and Switchos, a 6x2 valve switching device. A PepMap™ C18 column (300 µm X 15 cm, 100Å, 3 µm, LC Packings) was used with 0,05 % (v/v) trifluoroacetic acid (TFA) 5 % (v/v) acetonitrile (ACN) as solution A and 0.4 % TFA/80ACN as solution B, at a flow rate of 4 µl/min. The PepMap™ column was preceded by a µ-precolumn cartridge (C18 PepMap, 5µm, 100 Å, 300 um i.d.×5 mm) in a holder (LC Packings). The technical setup was controlled by the Ultichrom™ 3.1 software (LC Packings, Amsterdam, Netherlands). The solvents were continuously sparged with helium. Peptide trypsin digests were eluted from the column with a linear gradient of 5 % to 50 % solution B within 115 min. The wash and loading solvent used with the Famos autosampler to the µ-precolumn was 0.1 % TFA at a flow rate of 10µl/min from the outer pump.

The mass spectrometric analyses were performed on LC-interfaced Bruker Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray source set to the positive ion mode. The auto-MS/MS parameters were the following: scan range *m*/*z* 200–2000, scan speed 13000 *m*/*z* s−1, nebulizer flow 7 psi, dry gas flow 6.0 L/min, dry temperature

150°C, capillary −2 kV, skimmer 40V, ion charge control (ICC) target 11000, maximum accumulation time 200 ms, spectra averages 10, and rolling averages 5.

Protein identifications were performed by NCBInr and Swissprot Database searching, through Mascot, and MS/MS datasets were performed via BioTools™ 3.1 software (Bruker Daltonic, Matrix Science Ltd, UK). MS and MS/MS tolerance of respectively 0.5 Da and 1 Da and one missing cleavage site were allowed. Carbamidomethyl-cysteine and oxidation of methionine residues were considered in the fixed and variable modifications respectively. The resultant data was further evaluated by comparing the scores, the calculated and observed molecular mass and pI values, as well as the number of peptides matching to the protein and mostly, the percent sequence coverage.

3.5 Molecular inhibitors

DNA-dependent protein kinase (DNA-PK) inhibitor NU7441 and heat shock protein 90 (HSP90) inhibitor 17-AAG were purchased from TOCRIS Bioscience (Bristol, UK), and topoisomerase-I inhibitor (Topo-Ii) irinotecan from Mayne/Hospira UK Limited (Warwickshire, UK). DNA-PK potentiates the effect of radiation and some chemotherapeutics like doxorubicin and etoposide (Zhao et al., 2006). The 17-AAG inhibits HSP90 chaperon activity, oncogenic proteins such as $p185^{erbB-2}$, and has anti-tumor properties (Hostein et al., 2001; Schnur et al., 1995). Irinotecan prevents DNA replication and transcription by inhibiting topoisomerase-I enzyme.

3.6 Quantitative real-time polymerase chain reaction

Total DNA was extracted from M4A4-LM3 cells using the QIAamp DNA mini kit (Qiagen). PCR amplification was based on primers and probe targeting the E4 gene (Kanerva et al., 2002b). Human β-actin primers and probe were used as an internal control and to normalize the number of viral DNA copies for the amount of genomic DNA (Alvarez-Lafuente et al., 2007). The real-time PCR conditions for each 20-µl reaction were as follows: 2 x LightCycler480 Probes Master Mix (Roche), 500 nM each forward and reverse primer, 150 nM each probe, and 5 µl extracted DNA. PCRs were carried out in a LightCycler (Roche) under the following cycling conditions: 10 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 60°C, and 10 min at 40°C. All samples were tested in triplicate. A regression standard curve was established using DNA extracted from serial dilutions of pAd5easy plasmid (from 10^9 to 10^1 copies). Known amounts of human genomic DNA (800, 80, 8, 0.8, and 0.08 ng) were used to generate a standard curve for the β-actin gene.

3.7 Immunofluorescence staining and confocal microscopy

Phospho-γH2AX foci analysis was used to quantify double strand breaks (DSBs) induced by adenovirus, irradiation, and specific inhibitors: DNA-PK inhibitor (DNA-Pki) (NU-7441, 1 mM), HSP90 inhibitor (HSP90i) (17-AAG, 500 nM), or topoisomerase-I inhibitor (Topo-Ii) (irinotecan, 200 mM). The cells were plated on LabTek chambers and infected with Ad5-luc1. Finally, the cells were washed and fixed with 4 % paraformaldehyde for 10 min at room temperature and stored at 4 °C in phosphate-buffered saline. For DNA DSB quantification, the cells were immunostained with rabbit polyclonal anti-γH2AX and Alexa488 conjugated secondary antibody (Molecular Probes/Invitrogen). The cells were mounted with Vectashield with counterstain for nuclei with 4´,6 diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Cells were visualized using Zeiss LSM 5 Duo laser scanning confocal microscope (Jena, Germany). Analysis of γH2AX foci number and size were done with ImageJ software. In a single experiment, at least 40 cells from five independent fields were analyzed.

4. Treatment of patients with oncolytic adenoviruses (II, III, IV)

4.1 Advanced therapy access program (ATAP)

The Advanced Therapy Access Program was started in 2007 in partnership with the International Comprehensive Cancer Center Docrates, Helsinki, Finland. The purpose of this program is to offer experimental therapies to patients with metastatic cancers refractory to conventional therapies. ATAP is a personalized therapy program, not a randomized clinical trial. Altogether about 300 patients have undergone the oncolytic adenovirus therapy through this program so far. All treatments in this study were given in the context of a registered (ISRCTN 10141600) ATAP. ATAP is regulated by FIMEA (Finnish Medicines Agency) as determined by EC/1394/2007. Treatments were performed according to Good Clinical Practice and the Helsinki Declaration of World Medical Association (Article 35). FIMEA requires the reporting of treatment results and adverse events. Required reports to FIMEA have been provided as requested.

4.2 Patient selection and follow-up

Altogether 157 cancer patients were treated in three patient series (II-IV). All patients voluntarily contacted the clinic and the suitability of each patient for viral therapy was evaluated before treatment based on their medical history, clinical evaluation and other diseases. Detailed written information about the treatment was given to the patient by the oncologist and the patient signed the form of consent. Possible adverse events caused by the treatment were also explained and written information regarding this issue was provided to the patients and their caregivers.

Symptoms were assessed by interviewing the patient at each visit and by collecting information from medical records. Patients were monitored overnight at the hospital and thereafter as outpatients for a minimum 28 days post treatment. All adverse events (AE) were graded from 1 to 4 according to Common Terminology Criteria for Adverse Events (CTCAE) v3.0. Pre-existing symptoms were not listed as adverse effects if they did not become worse. However, if a symptom became more severe, for example, pretreatment grade 1 changed to grade 2 after treatment, it was scored as grade 2. The term “serious adverse event (SAE)” was used, if the adverse event was possibly or probably treatment related and it resulted in patient hospitalization, prolongation of hospitalization, life threatening situation or death.

The inclusion criteria were: solid tumors refractory to conventional therapies, progressive disease, WHO performance score ≤ 3 and no major organ function deficiencies. Exclusion criteria were: organ transplant, HIV or other major immunosuppression, brain metastasis, elevated bilirubin, ALT

or AST elevated more than three times above upper limit of normal, severe thrombocytopenia and other severe disease or organ malfunction.

4.3 Analysis of treatment efficacy

Tumor assessment by computer tomography (CT) or magnetic resonance imaging (MRI) was performed before treatment and again about six weeks later. Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse et al., 2005; Therasse et al., 2006) was applied to overall disease status including injected and non-injected tumors. RECIST categories are:

- Complete Response (CR): Disappearance of all target lesions
- Partial Response (PR): At least a 30 % decrease in the sum of the longest diameter of target lesions, taking as reference the baseline sum of longest diameter
- Stable Disease (SD): Neither sufficient for PR nor PD
- Progressive Disease (PD): At least a 20 % increase in the sum of the longest diameter of target lesions, or at least one new lesion

In addition to the standard criteria above, we used Minor Response (MR, 10-30 % reduction in the size of lesions) as an indicator of cases where biological activity might be present. Tumor density was evaluated according to the "Choi criteria" (Choi et al., 2007), which have been proposed useful in the context of oncolytic viruses (Park et al., 2008). Choi analysis is based on tumor size and density (Hounsfield unit) on CT and maximum standardized uptake value (SUVmax) on [18F]fluorodeoxyglucose positron emission tomography (FDG-PET). Choi *et al* proposed criteria where a decrease in tumor size of more than 10% or a decrease in tumor density of more than 15% on CT had a sensitivity of 97% and a specificity of 100% (Choi et al., 2007).

4.4 Quantitative real-time polymerase chain reaction for patients

Total DNA was extracted from serum by adding 3 µg of carrier DNA (polydeoxyadenylic acid; Roche, Mannheim, Germany) to 400 µl of serum and using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany). Extracted DNA was eluted in 60 µl nuclease-free water and DNA concentration was measured by spectrophotometry. PCR amplification was based on primers and probe targeting the E1A region flanking the 24 bp deletion (forward primer 5´-TCCGGTTTCTATGCCAAACCT-3´, reverse primer 5´-TCCTCCGGTGATAATGACAAGA-3´ and probe onco 5´FAM-TGATCGATCCACCCAGTGA-3´MGBNFQ). In addition, a probe complementary to a sequence included in the 24 bp region targeted for deletion was used to test the

samples for the presence of wild-type adenovirus infection (probe wt 5´VIC-TACCTGCCACGAGGCT-3´MGBNFQ).

The real-time PCR conditions for each 25 µl reaction were as follows: 2X LightCycler480 Probes Master Mix (Roche, Mannheim, Germany), 800 nM each forward and reverse primer, 200 nM each probe, and 250 ng extracted DNA. PCR reactions were carried out in a LightCycler (Roche) under the following cycling conditions: 10 min at 95 °C, 50 cycles of 10 s at 95 °C, 30 s at 62 °C, and 20 s at 72 °C and 10 min at 40 °C. All samples were tested in duplicate. TaqMan exogenous internal positive control reagents (Applied Biosystems, Carlsbad, CA, USA) were used in the same PCR runs to test each sample for the presence of PCR inhibitors. A regression standard curve was generated using DNA extracted from serial dilutions of the tested virus (1 x $10^{8\text{-}10}$ VP/ml) in normal human serum. The limit of detection and limit of quantification for the assay were 500 VP/ml of serum. All positive samples were further confirmed by real-time PCR using LightCycler480 SYBR Green I Master mix (Roche). Primers specific for each virus used in this study are listed below:

Table 11. List of primers.

Tested virus	Forward primer	Reverse primer	References
Ad5/3-cox2L-d24	5´-CACGTCCAGGAACTCCTCAG-3´	5´-CGGCCATTTCTTCGGTAATA-3´	(Pesonen et al., 2010)
ICOVIR-7	5′-GCGGGAAAACTGAATAAGAGG-3′	5′-CGGAGCGGTTGTGAACTG-3′	(Nokisalmi et al., 2010)
CGTG-102 a.k.a Ad5/3-d24-GMCSF	5′-GGAGTGCGCCGAGACAAC-3′	5′-ACTACGTCCGGCGTTCCAT-3′	(Koski et al., 2010)
Ad5-RGD-d24-GMCSF	Ad5-a forward: 5′- ACAAACGCTGTTGGATTTATGC-3′	RGD reverse: 5′-GATGGGCAGAAACAGTCTCC-3′;	(Pesonen et al., 2011)
	Ad5-b forward: 5′-AAACACCACCCTCCTTACCTG-3′	GMCSF reverse: 5′-TCATTCATCTCAGCAGCAGTG -3′)	
Ad5-d24-GMCSF	5′-AAACACCACCCTCCTTACCTG-3′	5′-TCATTCATCTCAGCAGCAGTG-3′	(Cerullo et al., 2010)
Ad3-hTERT-E1	5′-CATGATATCGTGCCAGCGAGAAGAGTTTT-3′	5′-CATTCTAGAGCGAGCACAATAGTTCTTTCA-3′	(Hemminki et al., 2011)

4.5 Cytokine analysis

Cytokine analysis was done with BD Cytometric Bead Array Human Soluble Protein Flex Set (Becton Dickinson) according to the instructions of the manufacturer. FCAP Array v1.0.1 software was used for data analysis. Patient's serum samples were used in 1:4 dilution.

4.6 Elispot analysis

Peripheral blood mononuclear cells (PBMC) were used to evaluate the induction of tumor and adenovirus specific immunity following treatment as reported (Cerullo et al., 2010; Koski et al., 2010). PBMCs were isolated by Percoll gradient. Cells were frozen in CTL-CryoABCTM serum-free media (Cellular Technology Ltd. Cleveland, Ohio). ELISPOT was performed according to MABtech manufacturer instructions (h-IFN-gamma ELISPOT PRO 10 plate kit). For adenovirus ELISPOT, cells were stimulated with the HAdV-5 Penton peptide pool (ProImmune, Oxford, UK). For the anti-tumor response survivin (BIRC5 PONAB), pool of CEA+Ny-ESO-1, pool of c-Myc +SSX2, MAGE-3 and WT-1 peptides were used (ProImmune). No pre-stimulation of PBMCs was done in order to avoid artificial or incorrect signals and to ensure adequate viability of cells which might be compromised during prolonged culture.

4.7 Neutralizing antibody titer determination

293 cells were seeded at a density of 1×10^4 cells/well on 96-well plates and cultured overnight. Serum samples were incubated at 56°C for 90 minutes to inactivate complement, and a 4-fold dilution series (1:1 to 1:16,384) was prepared in serum-free DMEM (Sarkioja et al., 2008). Ad5lucRGD (Kanerva et al., 2002b) was used for ICOVIR-7 and Ad5/3-luc1 (Kanerva et al., 2002b) for Ad5/3-cox2L-d24. Control viruses were mixed with serum dilutions and incubated at room temperature for 30 minutes. Cells in triplicates were infected with 100 VP/cell, and growth medium with 10 % FCS was added 1 hour later. Twenty-four hours post infection, cells were lysed and luciferase activity was measured (Luciferase Assay System, Promega; TopCount Luminometer, Perkin-Elmer). Luciferase readings were plotted relative to gene transfer achieved with Ad5lucRGD alone. The neutralizing antibody (NAb) titer was determined as the lowest degree of dilution that blocked gene transfer by more than 80 % (Pesonen et al., 2009).

5. Ethical aspects and considerations

Human samples: Blood samples were collected to monitor the safety of treatment and also for treatment efficacy analysis. This caused additional but acceptable harm for patients. Tumor samples were collected and analyzed for cox2 status in study II but this caused no extra harm to patients because tumor tissue samples were already available due to prior standard surgical resection. Ascites samples were gathered during ascites removal in order to relieve patients' symptoms. All samples were processed anonymously and used only for intended purposes. Treatment protocols were approved by the Surgical Ethics Committee of Helsinki University Central Hospital and fulfilled the legal requirements.

Radiological analysis: To monitor radiological responses CT or PET-CT scans was taken when suitable and this caused additional but acceptable ionizing radiation dose to patients. MRI imaging caused no biological harm to patients.

6. Statistics (I-IV)

In Study I, luciferase and GFP expression were analyzed with a two-tailed t-test. In Study II, correlations between baseline neutralizing antibody titer and viral genomes in the serum and between viral dose and transaminase increases were analyzed with Pearson correlation analysis. Non-parametric Spearman's Rho analysis was used to evaluate correlations between archival tumor Cox-2 expression and viral genomes in the serum, and between route of administration and adverse events. In Study IV, a two tailed t-test was used to assess significance in T-cell phenotype data, while non-parametric one-way ANOVA, with Dunn's multiple comparisons post-test was used for cytokine data. Responses and correlation to ELISPOT data were analyzed with Chi^2- and Mann-Whitney-U tests. Anti-tumor and anti-viral T-cell induction was calculated with two-tailed Fisher's exact test. In Studies II, III and IV, patient survival data was processed with Kaplan-Meier analysis and Log-Rank test (Study IV). All analyses were done with SPSS 15.0 software for Windows and a p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

1. Preclinical results (I)

1.1 The combination of radiotherapy and adenoviral gene therapy

Radiotherapy is important in the treatment of prostate cancer and breast cancer and it is widely used in many other malignancies. Prostate and breast cancer are the most common cancers worldwide. Because of this clinical relevance, we studied the combination of radiotherapy and adenoviral gene therapy *in vitro* using a prostate cancer cell line (PC-3MM2) and a breast cancer cell line (M4A4-LM3). In one experiment, lung cancer cell line LNM35/eGFP was also used.

Radiation can increase adenoviral transgene expression both *in vitro* and *in vivo*. However, the exact molecular and cellular mechanisms underlying this effect have remained largely elusive (Hingorani et al., 2008b; Vereecque et al., 2003; Zhang et al., 2003a). These mechanisms are clinically relevant, because radiation can enhance the expression and effect of therapeutic transgenes such as interleukins or GM-CSF. There is evidence that some modifications in viral structure might hinder or change the effect in an unfavourable way. Hingorani *et al* reported that radiation increased GFP expression only under the Egr-1 promoter, but not with the CMV promoter (Hingorani et al., 2008a) and the effect was cell line dependent.

These findings created a need to study these aspects more carefully. We have focused, in this study, on investigating the synergistic mechanisms only *in vitro,* because most of the unresolved questions remain at the cellular level.

1.2 Radiotherapy increases adenoviral transgene expression, regardless of the transgene, promoter, cancer cell line, or radiation dose

M4A4-LM3 and PC-3MM2 cells were radiated with 8 Gy single dose and infected with five different replication-deficient adenovirus vectors 24 h post irradiation. Adenoviruses were capsid modified, allowing us to further study the role of capsid modification in transgene expression. In this experiment luciferase was used as a transgene and its activity was measured 24 h post infection. As a conclusion, radiation enhanced luciferase expression in both cell lines, with all tested viruses and all concentrations compared to nonradiated controls (see Figure 1 and Supplementary Table 1 in study I).

To study whether the enhancement of transgene expression is promoter specific, radiated PC-3MM2 cells were infected with viruses with identical capsids but different promoters (one virus promoter: cytomegalovirus (cmv) and four human promoters: multi-drug resistance (mdr), α-lactalbumin (ala), cyclo-oxygenase 2 (Cox2), and vascular endothelial growth factor (VEGF)).

Radiation enhanced luciferase expression with all tested promoters (see Figure 2, panel A and Supplementary Table 1 in study I), except the vascular endothelial growth factor promoter. This promoter was further studied in two additional cell lines (M4A4-LM3 and LNM35/eGFP) Statistically significant enhancement was seen in M4A4-LM3 cells (see Figure 2, panel B in study I). These results suggest that many different types of promoters are susceptible to induction by radiation.

We also measured the expression of GFP to see if the effect of radiation depended on the reporter transgene. PC-3MM2 cells were radiated with 8 Gy dose and infected with two different viruses expressing GFP (Ad5-GL and Ad5-pk7-GL). Flow cytometric analyses showed that also GFP expression was enhanced similarly as luciferase expression (see Figure 2, panel C in study I). Overall, average radiation induced enhancement of transgene expression was 146 % (range: 0 % − 274 %).

Finally, the impact of radiation dose on luciferase expression was tested in M4A4-LM3 cells. Radiation increased luciferase expression with all tested doses (dose range: 0.5 Gy – 15 Gy) (see Figure 2, panel D in study I). It is interesting to note that the 2 Gy dose, which is an important dose from a clinical perspective, increased transgene expression significantly (+ 110 %) yet the higher doses seemed to be slightly more effective (maximum effect + 136 % at 12 Gy).

Our results are in line with previous results published by Hingorani *et al.* They showed that NIS, GFP, β-galactosidase (lacZ) and luciferase expression was increased due to radiation. These results were obtained in colorectal, head and neck and lung cancer irrespective of promoter (CMV, RSV, hTR, hTERT) and were also confirmed *in vivo* (Hingorani et al., 2008b).

1.3 Radiation upregulates HSP90 and both RNA and protein production

The effect of radiation in all cell types is quite unspecific. Reactive oxygen species can damage many cell organelles and disturb the global cell microenvironment as well as alter cell metabolism (Hallahan, 1996). Based on this, we used mass spectrometry analysis to find possible differences between irradiated and non-irradiated cancer cells that might contribute to the findings above.

M4A4-LM3 cells were radiated with an 8 Gy dose and 24 h post radiation, mass spectrometry analysis was performed. The analysis revealed the up-regulation of HSP90 in irradiated cells (see Supplementary Table 2 in study I). The up-regulation of heat shock proteins by cell stress, such as radiation is well known and it prompted us to study further the role of HSP90 in the context of adenoviruses and radiation.

We also studied radiation induced alterations in cell metabolism and protein production. M4A4-LM3 and PC-3MM2 cells were exposed to an 8 Gy dose which resulted in higher total protein

levels in irradiated cells measured at 24 h after radiation (see Figure 4, panel B in study I). In the M4A4-LM3 cell line, the total RNA levels were quantified also 24 h and 48 h after radiation. RNA levels increased 149 % and 71 %, respectively (see Figure 4, panel A in study I). Flow cytometric analysis was used to measure the effect of radiation on cellular GFP expression in M4A4-LM3 and LNM35/eGFP cells. In both cell lines, radiation enhanced cellular GFP levels (see Figure 4, panels C and D in study I).

In summary, it seems that radiation upregulates specific proteins like HSP90, but probably there are also unspecific changes in cell metabolism and protein production that might contribute also to the expression of foreign genes, such as genes from adenovirus vectors. Our unpublished results indicate that when M4A4-LM3 cells were exposed to heat shock, there was no effect on adenovirus transgene expression contrary to radiation (Figure 9). Zacal *et al* reported similar results in colon carcinoma (Zacal et al., 2005). This data supports the hypothesis that radiation alters cell microenvironment in such a way that it favors adenovirus gene expression and the effect cannot be obtained by any random stress to cancer cells.

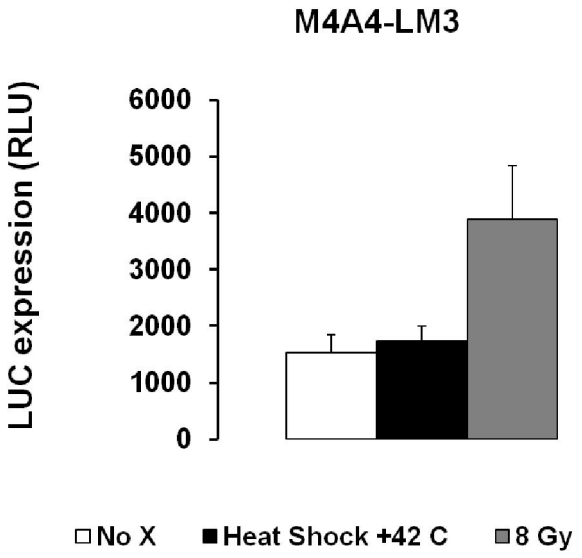

Figure 9. M4A4-LM3 cells were exposed to heat shock (that is 4 h at + 42 °C) and then infected 4 h later with Ad5luc1 (200 VP/cell). No increase was seen in luciferase levels compared to nonradiated controls. However, in the same experimental design, an 8 Gy dose increased luciferase expression by 154 % ($p < 0.001$). Error bars = standard deviation.

1.4 Radiation does not increase virus transduction or viral receptors

It has been suggested that radiation up-regulated gene expression could be explained by the increased virus transduction to cancer cells due to increased adenovirus receptors. One of the proposed effectors is upregulated Dynamin 2. Endocytosis of adenovirus to clathrin-coated vesicles requires the action of the large GTPase dynamin as a constrictase (Takei et al., 1995). There are reports in different cancer cell lines such as pancreatic and colon carcinoma that radiation upregulates dynamin 2 expression, leading to increased adenovirus uptake and increased adenovirus transgene expression (Egami et al., 2008; Qian et al., 2005). Qian *et al* also proposed that CAR does not contribute to increased transduction. Conflicting findings were reported in colorectal, head and neck and lung cancer where CAR and integrin-α levels increased after radiation (Hingorani et al., 2008b). However, radiation also exerts other nontransductional effects. There is evidence that adenovirus receptor expression might be cell cycle dependent, possibly explaining these conflicting results. Both CAR and α_v-integrin receptor showed increased cell surface availability in M-phase (1.5-fold and 2- to 3-fold increases, respectively) (Seidman et al., 2001). This result also raises a new opportunity to enhance adenovirus transgene expression by synchronizing cells to M-phase with the clinically available chemotherapeutic paclitaxel.

In our study the levels of CAR, CD46, heparan sulfate proteoglycans, integrin β_3 and integrin β_5 were measured by flow cytometry. We could not detect any changes in the cell surface receptor levels challenging some of the previous results (see Supplementary Figure 1 in study I). Viral DNA content of cancer cells at 24 h after radiation has been reported to be significantly higher than that of nonradiated (Egami et al., 2008). Contrary to that, our findings showed unchanged viral DNA content at 2 h and 24 h after infection regardless of radiation status (see Figure 3 in study I). In brief, our data support the hypothesis that radiation does not affect adenoviral transduction or receptor availability, but the mechanism is related to post-transductional events.

1.5 Enhancement of transgene expression is mediated through genotoxic stress regulation and repair

The radiation-induced enhancement of adenovirus transgene expression was previously explained by alterations in receptors relevant for adenovirus transduction. However, during recent years the focus in this research area has been in radiation induced DNA damage and further cellular signal transduction that favours adenovirus transgene expression. Many of these signal transduction pathways belong to the mitogen activated protein kinase (MAPK) superfamily and multiple MAPK pathways are activated following radiation and other toxic stress (Dent et al., 2003).

Increased adenovirus transgene expression has been reported after exposure of cells to various DNA damaging agents, such as cisplatin and N-acetoxy-acetylaminofluoride (Park et al., 2002; Zacal et al., 2005). The inhibition of radiation induced DNA damage repair by targeting DNA-dependent protein kinases (DNA-pk) is also associated with increased transgene expression from adenoviral vectors (Hingorani et al., 2008a).

The compact and supercoiled DNA double helix requires topological modifications during cellular processes such as transcription, replication, and repair (Gilbert et al., 2012). These modifications are executed by topoisomerase enzymes, and topoisomerase inhibitors such as topotecan and irinotecan have been widely used in the treatment of colorectal cancer (Gilbert et al., 2012). Topoisomerase inhibitors can also potentiate the effect of radiotherapy by inhibiting DNA damage repair (El-Khamisy et al., 2007). Zamir *et al* reported that adenoviruses induced host topoisomerase activity and inhibition of topoisomerase-I by topotecan enhanced adenovirus transgene expression in hepatic cells, colon cancer cells and prostate cancer cells (Zamir et al., 2007).

HSP90 is an ATP-dependent molecular chaperone that regulates the late-stage maturation, activation and stability of a diverse range of client proteins including protein kinases (Pearl et al., 2008). Although HSP90 is highly expressed in normal cells, where it helps to maintain protein homeostasis, HSP90 is also recruited by cancer cells to help mutated oncoproteins and buffer cellular stresses induced by oncogenesis (Neckers and Workman, 2012). HSP90 overexpression is related to poor prognosis (Pick et al., 2007) and it plays a versatile role by regulating a large number of cellular kinases and transcription factors (Ciocca and Calderwood, 2005). HSP90 inhibitors (e.g. tanespimycin, 17-AAG) are clinically very interesting because they sensitize tumor cells to radiation (Machida et al., 2003) and many of them are being tested in clinical trials with promising results (Modi et al., 2011; Neckers and Workman, 2012).

All these data support the hypothesis that radiation-induced transgene expression is mediated by persistent DNA damage and genotoxic stress regulation. Several factors (e.g. DNA dependent protein kinase inhibitors (DNA-PKi), topoisomerase-I inhibitors (Topo-i) or HSP90 inhibitors (HSP90i)) associated with DNA damage might also contribute to adenoviral transgene expression enhancement with radiation and thus we wanted to clarify this issue further.

DNA damage repair inhibitors combined with radiation were studied to evaluate the effect of genotoxic stress modulation on adenoviral transgene expression in M4A4-LM cells. Immunofluorescence analysis for phosphorylated histone H2AX (γH2AX) foci was used as a marker of double strand break (DSB) repair.

HSP90i increased radiation-induced DNA damage (see Figure 5, panel G in study I), but there was no correlation between DNA damage and transgene expression. Actually HSP90i decreased

adenovirus transgene expression regardless of radiation status (see Figure 6 in study I). It is possible that cellular and perhaps mutated heat shock proteins in M4A4-LM3 cells try to maintain oncogenesis (e.g. interfere with p53), but most likely HSP90i is anyhow able to counteract this at least partly, leading to down-regulation of adenoviral proteins. From a clinical point of view, it seems that the combination of adenoviruses and heat shock protein inhibitors is not favourable in the context of therapeutic transgenes.

Topo-i increased adenovirus transgene expression with and without radiation by +295 % and 134 %, respectively (see Figure 6 in study I). Topo-i increased also the number of DSBs with virus, but there was no additional effect with radiation (see Figure 5, panel F in study I).

DNA-PKi alone had only a modest effect to transgene expression (+11 %), but it greatly enhanced transgene expression with radiation (+499 %) (see Figure 6 in study I). Also, a previously unreported correlation between the number of DSBs and adenovirus transgene expression was detected (see Figure 5, panels F and G in study I). This result suggests that there is a relation between DNA damage and adenovirus transgene expression and this relation can be modified with inhibitors involved in DNA damage repair. The most probable signal transduction pathway that links these findings is the MAPK pathway. However, p38/mitogen activated protein kinase inhibition did not enhance adenovirus transgene expression (Hingorani et al., 2008a). Thus, more studies are needed to specify the roles of different MAPK pathways *in vivo*.

All these data together indicate that radiation has various different effects that contribute in concert to the enhanced transgene expression from adenovirus vectors. Perturbations in cell metabolism and protein production induce additional alterations in intracellular signaling environment that favours adenovirus gene expression. Specifically, MAPK/ERK and PI3K pathway signaling have been implicated. Radiation also induces a transcriptional effect via the NF-κB pathway by altering transcription of genes involved in cell cycle arrest and DNA repair (Vereecque et al., 2003). In addition, radiation has been shown to increase genetic recombination facilitating adenoviral transgene expression (Gridley and Slater, 2004; Stevens et al., 2001). Radiation induced genetic hyper-recombination which was rather long-lasting up to three days, and thus facilitated viral gene expression (Stevens et al., 2001). Adenoviral genes from the E4 region also have properties that favor adenovirus transgene expression even without radiation and it is possible that also this feature is enhanced by radiation (Weitzman and Ornelles, 2005). However these E4 region dependent functions require at least in part the E1B55k protein, which is not present in E1 deleted viruses that were used in this study.

1.6 Translational relevance and limitations

Radiation has a positive effect on adenovirus transgene expression both *in vitro* and *in vivo* in various tumor cell types. The combination of radiation and adenovirus gene therapy suggests additional or even synergistic effects. In study I, the combination of radiotherapy and adenovirus gene therapy revealed a possible synergy mechanism with chemotherapeutics as well. This was also first time according to our knowledge, when a HSP90 inhibitor, DNA-PK inhibitor, and topoisomerase inhibitor were studied together with radiation and adenoviruses. Based on these results, it would be rational to combine adenoviruses with radiotherapy in the treatment of prostate or breast cancer. Radiotherapy could enhance therapeutic transgene expression from intratumorally injected adenovirus vectors. Because in this setting both treatments are local, the adverse events reactions could be better tolerated, but local anti-tumor effects could be enhanced. Intratumoral injection to prostate and breast tissue is rather easy and practically possible. The combination could be further improved by agents preventing DNA damage repair, such as DNA-pk inhibitors.

There are also some limitations in this study. Radiotherapy is usually given in small daily doses (fractions) and thus the whole treatment protocol takes several weeks. In this study we have not studied the effect of fractionated radiotherapy and this significantly limits the translation of these results to clinical reality. For example, a typical breast cancer radiotherapy protocol takes five weeks (total dose 50 Gy, given in 2 Gy fractions per day) and it is not known how adenoviruses should be administered in this context and how fractionated radiation affects adenovirus transgene expression. There are technical and practical problems that prevent us from performing an *in vivo* experiment. For example, mice cannot be irradiated daily, the immobilization of mice during irradiation is not possible, which limits the dose delivery to tumors. Finally, we have studied transgene expression only from replication-deficient adenovirus vectors, but oncolytic/conditionally replicative adenoviruses would be even more relevant. Probably there are no major differences between replication-deficient and oncolytic adenoviruses regarding transgene expression. In theory, transgene expression from oncolytic adenoviruses (e.g. E1A deleted) should be more effective due to viral replication and an intact E1B55k/E4orf6 complex that potentiates the DNA damage induced by radiation.

2. Clinical results (II, III, IV)

2.1 Adenovirus therapy shows good safety in cancer patients

In study II the safety of Ad5/3-cox2L-d24 was studied in 18 patients (see Table 2 in study II). In study III the safety of ICOVIR-7 was studied in 21 patients (see Table 2 in study III) and finally in more versatile design, the safety of various oncolytic viruses was studied in 124 patients in Study IV (see Table 2 and Supplementary Table 1 in study IV). All treatments were performed as part of the Advanced Therapy Access Program (ATAP) as described in Material and Methods (see section 4.1). Note, that ATAP is not a clinical trial. In studies II and III, one oncolytic virus as a single therapeutic agent was used for each patient cohort and in study IV, several oncolytic viruses were used as a single therapy or serial therapy. Altogether 157 patients with advanced metastatic tumors were treated and in total six different oncolytic virus constructs were used.

The most common detected adverse events are summarized in Table 12. Adverse events are categorized as hematological adverse events, known also as non-clinical adverse events because most of them are laboratory findings without any clinical symptoms or known clinical relevance. Clinical adverse events are findings that cause clinical symptoms for patients and may need medical intervention.

The most common detected grade 1 or 2 hematological adverse events were (Table 12): anemia (36.1 – 81.0 %), leukocytopenia (22.2 – 100.0 %), and elevated liver enzymes (39.2 – 77.8 %). The most common detected grade 1 or 2 clinical adverse events were (Table 12): injection or tumor site pain (4.8 - 28.6 %), any other pain (83.3 – 100.0 %), nausea or vomiting (38.1 – 90.5 %), fever (50.0 – 90.5 %), and fatigue (55.6 – 78.4 %). No grade 4 adverse events were seen in studies II and III, but in study IV, six patients had grade 4 adverse events (Table 12). The most likely explanation for this is a higher number of enrolled patients.

Hazard analysis was done for each adverse event. The term serious adverse event (SAE) was applied if the adverse event was possibly or probably treatment related and it resulted in patient hospitalization, prolongation of hospitalization, life threatening situation or death. Altogether there were thirteen serious adverse events in eleven different patients (Single treatment: six patients, Serial treatment: four patients, both treatments: one patient in study IV (see Supplementary Table 3 in study IV and Table 12). No treatment related deaths occurred in any studies. In brief, adenovirus gene therapy with different virus modifications or multiple dosing was safe.

It has been proposed that cytokines might be able to predict harmful inflammation and elevated levels are known to be associated with systemic inflammatory response syndrome (SIRS) and may induce a multi organ failure (MOF) (Brunetti-Pierri et al., 2004; Raper et al., 2003). In our study,

blood inflammatory cytokine level analysis was performed for each patient. IL-6, IL-8 and TNF-α were selected for safety analysis due their pro-inflammatory role (Mistchenko et al., 1994; Stenvinkel et al., 2005) and IL-10 because of its anti-inflammatory nature. The analysis did not reveal high cytokine levels in any of the studies emphasizing the favourable safety profile of these adenovirus treatments.

All adenovirus cancer gene therapy trials carried out throughout the world so far have shown good safety and thousands of patients have been enrolled. No treatment related deaths have been reported. For comparison, just in one monoclonal antibody trial (Ibilimumab), 14 treatment related deaths occurred (2.1 % of patients) (Hodi et al., 2010). The most common adverse events in adenovirus trials have been flu-like symptoms (fever, fatigue, chills/rigors, nausea/vomiting) and also mild to moderate pains are usually seen. The safety profile in our studies is consistent with these reports.

The primary aim in study IV was to evaluate the safety of serial treatment (= three rounds of adenovirus treatment within 10 weeks) compared to a single therapy. In single therapy groups grade 4 adverse events were seen in 2.6 − 2.8 % of patients and in 3.9 – 4.8 % of patients with serial treatment, and serious adverse events 5.6 – 7.7 % vs. 4.8 − 7.8 %, respectively. These data suggest that serial treatment is as safe as a single round of therapy. However, the overall probability for grade 3 and grade 4 adverse events was slightly higher in certain serially treated cohort (grade 3: 23 % vs. 33 % and grade 4: 3 % vs. 5 %, see Supplementary Table 2 in study IV). This is explained by the accumulation of adverse events during repeated dosing.

Table 12. Summary of the most common detected adverse events according to CTCAE v3.0 criteria

	Study II	Study III	Study IV				
Adverse events	**Single (N = 18)**	**Single (N = 21)**	**Single (N = 39)**	**Serial (N = 21)**	**All CGTG-102 (N = 115)**	**Single (N = 72)**	**Serial (N = 51)**
Hematological (gr. 1 - 2):							
anemia	50.0 %	57.1 %	61.5 %	81.0 %	42.6 %	36.1 %	45.1 %
leukocytopenia	22.2 %	NA	87.1 %	100.0 %	48.7 %	47.2 %	43.1 %
elevated liver enzymes	77.8 %	61.9 %	51.3 %	71.4 %	42.6 %	40.3 %	39.2 %
Clinical (gr. 1 - 2):							
injection site pain/tumor	16.7 %	4.8 %	12.8 %	28.6 %	26.1 %	22.2 %	27.5 %
other type of pain	83.3 %	100.0 %	100.0 %	100.0 %	100.0 %	100.0 %	100.0 %
nausea, vomiting	61.1 %	38.1 %	46.2 %	90.5 %	57.4 %	38.9 %	74.5 %
fever	50.0 %	85.7 %	89.7 %	90.5 %	81.7 %	72.2 %	82.0 %
fatigue	55.6 %	76.2 %	71.8 %	76.2 %	72.2 %	59.7 %	78.4 %
Hematological (gr. 3):							
anemia	0.0 %	4.8 %	0.0 %	4.8 %	3.5 %	4.2 %	2.0 %
leukopenia	0.0 %	0.0 %	23.1 %	52.4 %	16.5 %	23.6 %	3.9 %
elevated liver enzymes	0.0 %	0.0 %	7.7 %	9.5 %	5.2 %	5.6 %	3.9 %
Clinical (gr. 3):							
injection site pain	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %
other type of pain	0.0 %	0.0 %	2.6 %	0.0 %	7.0 %	8.3 %	3.9 %
nausea, vomiting	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0%
fever	0.0 %	0.0 %	0.0 %	4.8 %	2.6 %	1.4 %	3.9 %
fatigue	0.0 %	0.0 %	0.0 %	0.0 %	3.5 %	2.8 %	3.9 %
All grade 4 (no. of patients)	0	0	1 (2.6 %)	1 (4.8 %)	4 (3.4 %)	2 (2.8 %)	2 (3.9 %)
Serious adverse events # (no. of patients)	0	0	3 (7.7 %)	1 (4.8 %)	7 (6.1 %)	4* (5.6 %)	4* (7.8 %)

Serious adverse event analysis include all grades (1 – 4)

* One patient reported in both groups

2.2 Neutralizing antibodies and virus in circulation

Neutralizing antibody titer measurements were used to evaluate immunological responses in the patients following the adenovirus treatment. In addition, viruses in the circulation were measured by quantitative real time polymerase chain reaction (qPCR).

Neutralizing antibody measurements were done for all patients in studies II and III, except one patient in study II (sample was not available) (Table 13). More than half of the patients in both studies (82.4 % and 57.1 %, respectively) had positive antibody titers before treatment, indicating natural pre-existing immunity (Table 13). In study II, Ad5/3-cox2L-d24 induced increased antibody titers for all patients, whereas ICOVIR-7 induced increasing titers for 76.2 % of patients. These lower numbers of increased titers seemed to correlate with lower pre-existing antibodies for ICOVIR-7. It remained unclear why the number of positive pre-treatment patients was lower for ICOVIR-7, since there were no major differences in patients' pre-treatment profiles.

All patients in studies II, III and IV were negative for viral genome in circulation before treatment, as expected. After the treatment, most of the patients (range: 83.3 - 94.7 %) were positive for virus in blood (Table 13). The highest detected virus concentration was seen in a patient with prostate cancer (see Table 4 in study II). This patient belonged to the highest dose cohort of Ad5/3-cox2L-d24 (3×10^{11} VP) and was highly positive for cox2L expression based on the pre-treatment analysis of the tumor block, suggesting effective viral replication. The viral replication in patients is anyhow a debatable issue because the highest detected total viral load (if blood volume 5000 ml → total virus load was 2.63×10^{10} VP) was 10-fold less than the total injected dose (3×10^{11} VP). However, high viral concentration did not impact patient's survival (44 days) regardless of stable disease response based on tumor marker analysis (see Table 4 in study II). Interesting results were seen in study IV, where internal survival comparison was possible based on study design (see next section 2.4). There were more viral genome positive patients in the single treated group (94.7 %) compared to the serially treated patients (85.0 %) (Table 13), but survival was better for serially treated (Table 14). The high viral concentrations were detected both in serial and single treatment patients. There were no differences in the mean or median titers (see Table 1A in study IV).

In conclusion, the highest viral concentrations were seen usually 2-4 days after treatment and were detectable in some patients for several weeks. The frequency of post-treatment virus positive serum samples seemed to increase with the injected viral dose.

In an earlier phase I/II prostate cancer trial, all patients developed antibodies to oncolytic adenovirus CG7870, but no complete or partial responses were seen based on PSA levels (Small et al., 2006). Five out of twenty-three patients had decreased PSA levels, resulting in 21.7 % disease control. Small *et al* also reported interesting "secondary" or "delayed" peaks for virus presence in

plasma typically between day 2 and day 8, suggesting virus replication in 70 % of the patients. Nevertheless, our data and most other trials support the conclusion that usually the best treatment responses are seen with high injected viralk doses and high injected doses correlate with high detected doses.

Table 13. Summary of adenovirus antibody titers and qPCR data detecting adenovirus genome in circulation in studies II, III and IV.

	Neutralizing antibodies	**qPCR**
Study II (N = 18)	*Number of evaluable patients:* *17*	*Number of evaluable patients:* *18*
Single (N = 18)	**Pre-treatment baseline:** 14 positive (82.4 %) **Increasing titers:** 17 patients (100.0 %) **Highest titer:** 16384	**Pre-treatment baseline:** all negative **Post-treatment:** 15 positive (83.3 %) **Highest concentration:** 5 259 766 VP/ml
Study III (N = 21)	*Number of evaluable patients:* *21*	*Number of evaluable patients:* *21*
Single (N = 21)	**Pre-treatment baseline:** 12 positive (57.1 %) **Increasing titers:** 16 patients (76.2 %) **Highest titer:** 16384	**Pre-treatment baseline**: all negative **Post-treatment:** 18 positive (85.7 %) **Highest concentration:** 4 038 049 VP/ml
Study IV (N = 124)	*Number of evaluable patients:* *0*	*Number of evaluable patients:* *single 38, serial 20*
Single (N = 39)	*NA*	**Pre-treatment baseline:** all negative **Post-treatment:** 36 positive (94.7 %) **Highest concentration:** 72 613 VP/ml
Serial (N = 21)	*NA*	**Pre-treatment baseline:** all negative **Post-treatment:** 17 positive (85.0 %) **Highest concentration:** 611 235 VP/ml
CGTG-102 (N = 115)		
Single (N = 72)	*NA*	*NA*
Serial (N = 51)	*NA*	*NA*

NA = data not available, VP = virus particle

2.3 Evidence of immunological T-cell activity

In study IV, we also evaluated T-cell responses against tumor and adenovirus by ELISPOT for both single and serially treated patients. In the first part, 31 % of single treated patients had increased tumor-specific $CD8^+$ T-cells, whereas increased $CD8^+$ T-cells were observed in 43 % of serially treated patients, showing more T-cell induction for serially treated patients. The corresponding numbers for decreased T-cells in blood were 28 % and 43 %, respectively. Decreased $CD8^+$ T-cells might indicate T-cell trafficking to tumor and positive response to adenovirus treatment. So, less anergy (no induction or trafficking) was seen for serially treated patients (see Figure 1 in study IV). Seventy two percent (28/39) of single treated and 90 % (19/21) of serially treated patients demonstrated increasing levels of Ad5-specific $CD8^+$ T-cells (see Supplementary Figure 3 in study IV). These data raise the possibility that immunological factors might contribute to treatment response and survival.

2.4 Evidence of anti-cancer efficacy

RECIST analysis (Therasse et al., 2005; Therasse et al., 2006) based on radiological response or measurements of tumor markers in the blood have been used to quantify the effect of a tested treatment in oncology. However, these methods are not optimal for various reasons and they are even less optimal for most modern therapies. A notable example was seen in a phase III clinical trial of sipuleucel-T (Provenge), a cell based immunotherapy for advanced prostate cancer, where only one out of the 341 patients in the active arm showed a partial response according to RECIST, and only 2.6 % of patients had $\geq$ 50 % reduction in PSA levels. Still, a 4.1 month improvement in the median survival (25.8 months vs. 21.7 months) was achieved and the drug was approved by FDA in April, 2010 (Kantoff et al., 2010; Mellman et al., 2011). Progression free and overall survival is considered the most accurate method to monitor treatment efficacy. In addition to these efficacy indicators, attention in oncology trials are given also to other aspects of treatment suitability like quality of life, adverse event profile and the costs of treatment.

Efficacy analysis of oncolytic viruses is very challenging. First, local viral replication in tumors might cause inflammation that enlarges the tumor, leading to incorrect radiological interpretation of disease status. This phenomenon is sometimes called pseudoprogression. Both PET-CT and MRI imaging are sensitive for inflammation too, so they are unlikely to solve this problem. Secondly, lytic processes in tumor cells due to virus replication might release tumor markers to the circulation and cause false positive marker responses. Thirdly, there are no generally accepted and reliable methods to measure other clinically relevant responses, such as reduction of ascites or reduction of pleural fluid. Histological response evaluation is a method where tumor regression grading after

therapeutic intervention is evaluated by tissue samples (Rubbia-Brandt et al., 2007). This would be suitable and objective for adenovirus treatment as well, but histological response evaluation is possible only for neo-adjuvant treatment before surgical resection and this is not common in adenoviral cancer gene therapy.

Guidelines for the evaluation of immune therapy activity in solid tumors have been created and could be applied to adenovirus treatments (Wolchok et al., 2009). Four immunological response patterns have been detected and these results have been translated into new response criteria called the immune-related response criteria (irRC). The four response patterns in irRC classification are: (i) shrinkage in baseline lesions, without new lesions; (ii) durable stable disease (in some patients followed by a slow, steady decline in total tumor burden); (iii) response after an increase in total tumor burden; and (iv) a response in the presence of new lesions. All patterns were associated with favorable survival (Farolfi et al., 2012; Wolchok et al., 2009).

The disease control (=stable disease or better) according to RECIST was 40.0 % with oncolytic adenovirus Ad5/3-cox2L-d24 in study II, and 41.7 % with ICOVIR-7 in study III (Table 14). Disease controls in these studies based on tumor markers were 70.6 % and 23.1 % and median survivals 107 days and 92 days, respectively. Due to the study design, there are no control groups in either of the study and the efficacy cannot be directly estimated. However, these results are promising, since all patients had a progressive tumor before viral treatment.

In study II, one patient with neuroblastoma showed partial response (71.1 % reduction in the tumor volume and 33.0 % reduction in the longest diameter) one month after treatment and his bone marrow was also free of disease for first time since diagnosis (see Figure 3 in study II). Bone marrow biopsies showed disease relapse 3 months later. In study III, a 9-year-old boy with Wilms tumor had partial response with a 37 % overall reduction in the sum of tumor diameters (see Figure 1 in study III). In addition, two patients in study II had long-lasting stable disease response, 93 days and 316 days, respectively. Besides, the tumor density of one patient decreased by 16 %, which might also indicate benefit (Park et al., 2008) based on Choi criteria.

In study IV, 124 patients were enrolled. In the first part of the study, 60 patients were treated once (= single therapy, 39 patients) or three rounds (= serial therapy, 21 patients). Altogether six different oncolytic viruses (Ad5/3-d24-GMCSF a.k.a CGTG-102, Ad5-d24-GMCSF, Ad5-RGD-d24-GMCSF, Ad5/3-cox2L-d24, ICOVIR-7 and Ad3-hTERT-EI) were used, making this patient population and the treatment protocol more heterogeneous than in the later part of the study, where we focused more on Ad5/3-d24-GMCSF (=CGTG-102 virus). In the latter part, altogether 115 patients were enrolled (including 51 patients from first part). Two major differences exist in study IV compared to studies II and III. First, serial treatments were not used in studies II and III.

Secondly, single treated patients were used as a control group for serial treated patients in study IV, but because the study design is not a randomized trial, all results should be interpreted carefully.

The radiological disease controls (=stable disease or better) in study IV for single treated patients were 40.9 % and 74.0 %, and for serial treated 50.0 % and 48.0 % (Table 14). Disease control measured by tumor markers were 38.1 % and 58.0 % for single treated, and 37.5 % and 48.0 % for serial treated (Table 14). Based on disease control results, single treatments were more effective and favorable. However, the survival data suggested benefit for serial treated patients yet the difference was not statistically significant (Table 14). In the first part, the survival difference was 141 days and in the latter part 166 days. Survival at 200 days was also better for serial treated patients. Twenty-two patients received three different viruses in a serial treatment in a first-in-humans application of sero-switching with oncolytic viruses (see Table 2 in study IV). The reason for sero-switching is the opportunity to avoid pre-existing neutralizing antibodies. Median survival for these patients was 241 days and therefore the theoretical advantage of avoiding neutralizing antibodies (Hemminki et al., 2002; Kanerva et al., 2002b; Raki et al., 2011; Sarkioja et al., 2008) did not manifest in the survival advantage. For patients with CGTG-102 only, the median survival was 291 days.

These results indicate that serial treatment might be more effective. As stated earlier, the survival and efficacy data showed here is only preliminary and should be interpreted with caution, because these studies are not randomized trials and other factors might also contribute to the results.

Table 14. Summary of anti-tumor efficacy observed in patient series.

Study Design	RECIST	Tumor Markers	Median Survival
Study II (N = 18)	*Number of evaluable patients: 5*	*Number of evaluable patients: 14*	
Single (N = 18)	PR: 1 (20.0 %) MR: 1 (20.0 %) SD: 0 (0.0 %) PD: 3 (60.0 %)	Number of markers: 17 PR: 2 (12.0 %) MR: 4 (23.5 %) SD: 6 (35.0 %) PD: 5 (29.5 %)	107 days
Study III (N = 21)	*Number of evaluable patients: 12*	*Number of evaluable patients: 10*	
Single (N = 21)	PR: 1 (8.3 %) MR: 2 (16.7 %) SD: 2 (16.7 %) PD: 7 (58.3 %)	Number of markers: 13 PR: 0 (0.0 %) MR: 2 (15.4 %) SD: 1 (7.7 %) PD: 10 (76.9 %)	92 days
Study IV (N = 124)	*Number of evaluable patients: single 22, serial 18*	*Number of evaluable patients: single 21, serial 8*	
Single (N = 39)	PR: 0 (0.0 %) MR: 1 (4.5 %) SD: 8 (36.3 %) PD: 13 (59.1 %)	PR: 1 (4.8 %) MR: 2 (9.5 %) SD: 5 (23.8 %) PD: 13 (61.9 %)	128 days 35 %*
Serial (N = 21)	CR: 1 (5.6 %) PR: 1 (5.6 %) MR: 2 (11.1 %) SD: 5 (27.8 %) PD: 9 (50.0 %)	CR: 0 (0.0 %) PR: 2 (25.0 %) MR: 1 (12.5 %) SD: 0 (0.0 %) PD: 5 (62.5 %)	269 days 57 %*
CGTG-102 (N = 115)	*single 23, serial 22*	*single 26, serial 29*	
Single (N = 72)	Disease control 74 %	Disease control 58 %	111 days 37 %*
Serial (N = 51)	Disease control 48 %	Disease control 48 %	277 days 58 %*
Summary of disease controls[#]:			
Study II	40.0 %	70.6 %	
Study III	41.7 %	23.1 %	
Study IV			
Single (N = 39)	40.9 %	38.1 %	
Serial (N = 21)	50.0 %	37.5 %	
Single (N = 72)	74 %	58 %	
Serial (N = 51)	48 %	48 %	

CR: Complete response, PR: Partial response (>30 % decrease), MR: Minor response (10 – 30 % decrease), SD: Stable disease (< 20 % increase), PD: Progressive disease (≥ 20 % increase or new lesions), # Disease control = stable disease or better, * survival at 200 days

2.5 The relevance of clinical studies and limitations of adenovirus therapy

According to our knowledge, ICOVIR-7 in study III was the first RGD-modified oncolytic adenovirus used to treat cancer patients. The first tropism-modified oncolytic adenovirus studied in a clinical phase I trial was Ad5-d24-RGD (Kimball et al., 2010). Twenty-one patients with recurrent gynecologic disease were treated daily for three days in this trial. Clinical adverse events were limited to grade 1 and 2 fever, fatigue and abdominal pain, no treatment related grade 3 or 4 adverse events were seen and the maximum tolerated dose was not reached. No complete or partial responses were seen, but 15 out of 21 patients had stable disease (= disease control 71.4 %). Based on tumor markers, disease control was observed in 7 patients (= disease control 33.3 %).

The safety profile of Ad5-d24-RGD was consistent with ICOVIR-7 in our study. The disease control reported by Kimball *et al* was higher than what we saw in study III (RECIST: 41.7 % vs. 71.4 % and tumor markers: 23.1 % vs. 33.3 %). The follow-up time in the phase I trial was only one month, probably explaining this difference, because in our study the control images were acquired about two months after treatment. Our study also included various different malignancies, whereas only gynecological cancers were included in phase I trial by Kimbal *et al* and this might also have an effect on the results.

Comparative analysis of different treatment protocols is still scarce. For example Small *et al* (Small et al., 2006) used a single intravenous infusion, Kimball *et al* (Kimball et al., 2010) used daily treatments for three days and Pan *et al* (Pan et al., 2009) used intratumoral injection once a week for eight weeks. Treatment protocols should be based on pre-clinical results, but translational problems, limitations in animal models and many practical issues play a role when treatment protocols are selected. Our results showed that serial treatment is as safe as single treatment and suggest that the treatment efficacy could be improved by multiple dosing.

The most extensive survival was seen in the serially treated cohort with CGTG-102 virus only (291 days). This virus encodes GM-CSF as a transgene, suggesting that immunological responses might be involved, at least partly, for the improved survival. In brief, based on clinical trial reports and our data, it seems that viral modifications are not that crucial in determining the safety or efficacy, but more attention should be aimed at interactions of the viruses and the host immune system.

Obviously the main limitation in all our clinical patient series is the fact that they are not randomized clinical trials (which would have more rigorous patient selection and predefined treatment protocol). Thus, these results should be verified eventually in randomized clinical trials. Actually, a clinical trial with CGTG-102, sponsored by Oncos Therapeutics Ltd (Helsinki, Finland)

is open and the first patients have been treated during summer 2012 (www.ClinicalTrials.gov, NCT01437280). This is the first oncolytic virus trial ever performed in North Europe.

Instead of focusing on one cancer type, we have treated various solid advanced tumors. Some might consider this heterogeneity as a confusing factor, but from a different perspective it can be considered as a strength. It is very promising that the treatments showed benefits for many patients regardless of their disease. It is also important to keep in mind that oncolytic adenoviruses have been designed and developed to work in various cancer types.

CONCLUSIONS AND FUTURE PROSPECTS

The modern viral cancer gene therapy era started roughly 25 years ago, but only two therapeutic agents have been approved for clinical use in China, even though many sophisticated viruses have been tested in various clinical trials with excellent safety and promising efficacy. There are probably many reasons for this rather slow progress and more efforts are needed to study the clinical validity of oncolytic virotherapy in treatment of cancer patients also in Europe and in U.S..

Insufficient funding and increasing complexity of pharmaceutical development limits the progress. It has been estimated that the development of one new pharmaceutical agent will take roughly 20 years and cost at least 500 million euro. Such a huge amount of money and resources do not exist in any university and thus the collaboration between commercial pharmaceutical companies and academic research groups should be improved and encouraged. Increasing regulations and juridical issues also complicate development, so some kind of reasonable balance should be found between limiting control and uncontrolled freedom. Also, the novelty of gene therapy has been sometimes more of a burden than a blessing and too high expectations are very difficult to meet (Sheridan, 2011).

It is still unknown how adenovirus gene therapy works eventually as a single therapy for less advanced cancer patients, but the efficacy has been unsatisfying so far. Nevertheless, adenovirus gene therapy seems promising and effective as a combination therapy underlined by the synergy with radiotherapy, chemotherapeutics and other anti-cancer modalities. This is supported also by our finding where radiation had a positive effect for adenovirus transgene expression *in vitro* in various tumor types. The combination of radiotherapy and adenovirus gene therapy revealed a possible synergistic mechanism with chemotherapeutics, giving a pre-clinical background for this approach. Based on our results it would be rational to combine adenoviruses with radiotherapy and the expression of therapeutic transgenes from adenoviral vectors could be enhanced in this setting leading to better tumor control.

The safety of treatments in our clinical patient series was favorable. Altogether there were thirteen (13) serious adverse events in eleven (11) different patients. So, severe adverse event probability per patients was 7.0 %, which can be considered rather low compared to conventional therapies. We studied six different oncolytic viruses in patients with advanced solid tumors, but there were no major differences between the viruses regarding their safety or efficacy. However, CGTG-102 (Ad5/3-d24-GMCSF), which encodes immunostimulatory GM-CSF was perhaps the most promising virus construct of the all six tested viruses. This evokes the possibility that viral

modifications are not crucial, but immunological responses in patients eventually determines the outcome.

More careful patient selection for adenovirus treatment based on analysis of tumor receptors (e.g. CAR or desmoglein 2) or other tumor properties (e.g. Cox2 expression) might also improve the treatment results in clinical trials. Treatment selection based on tumor properties is a common clinical practice in oncology for many anti-tumoral agents. For instance, mono-clonal antibodies such as Trastuzumab, are used for HER2 positive breast cancer only. However, it is known that tumor properties might change during the years and for example 25 % of metastases with HER2-positive breast cancer patients turn negative after treatment (Amir et al., 2012). Similar shifting might take place in receptors relevant for adenovirus, hampering transductional targeting or other specific modifications in vectors. Thus, pre-treatment properties must be interpreted with caution and repeated tumor property determination is sometimes needed.

Viral cancer gene therapy is a very challenging field, because expertise from many areas such as oncology, virology, and immunology is needed (Figure 10). Faster progress in the field of viral cancer gene therapy might take place if the fragmented knowledge could be combined more efficiently. Especially, the importance of immunology has become evident during recent years and probably immunological aspects will determine eventually the fate of the viral cancer gene therapy field. The question is: “which happens first, fast virus eradication because of immune response towards the virus, or the tumor eradication due to virus-induced immune response against tumor?” If the latter happens, success might follow.

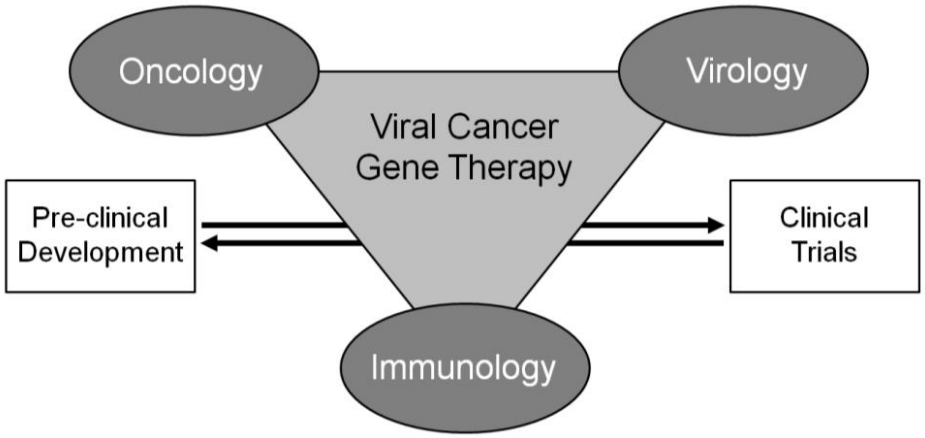

Figure 10. The dimensions of viral cancer gene therapy.

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